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EXHIBIT A

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Human Pancreatic Islet-Derived Progenitor Cell Engraftment in Immunocompetent Mice

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Running Title: Stem Cell-derived Xenografts in Immunocompetent Mice

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Abstract/Summary

The potential for the use of stem/progenitor cells for the restoration of injured or diseased tissues has garnered much interest recently, establishing a new field of research called regenerative medicine. Attention has been focused on embryonic stem cells derived from human fetal tissues. However, the use of human fetal tissue for research and transplantation is controversial. An alternative is the isolation and utilization of multipotent stem/progenitor cells derived from adult donor tissues. We have previously reported on the isolation, propagation, and partial characterization of a population of stem/progenitor cells isolated from the pancreatic islets of Langerhans of adult human donor pancreata. Here we show that these human adult tissue-derived cells, nestin-positive islet-derived stem/progenitor cells (NIPs), prepared from human adult pancreata survive engraftment and produce tissue chimerism when transplanted into immunocompetent mice either under the kidney capsule or by systemic injection. These xenografts appear to induce immune tolerance by establishing a mixed chimerism in the mice. We propose that a population of stem/progenitor cells isolated from the islets of the pancreas can cross xenogenic transplantation immune barriers, induce tissue tolerance, and grow.

Introduction

A revelation in the field of tissue regeneration has been the finding that pluripotent stem cells, or multipotent progenitor cells, exist not only in embryo blastocysts and fetal gonadal ridges but also exist in probably all organs of the adult body (1-8). Stem cells manifest at least four important properties: they are highly mobile, have the ability for self renewal, differentiate into different cell lineages given exposure to appropriate local environmental stimuli known as growth factors or morphogens (1), and may induce immune tolerance (9). Morphogens are typically provided by localized spatial regions of mesenchyme, so called mesenchymal niches. Many studies have been reported about how neural stem cells can be converted into blood (although controversial) (10), and hematopoietic stem cells can be converted into brain (11, 12), muscle (13), heart

(14) and liver (15, 16). It also has been shown that pancreas-derived stem cells can become liver (17) and liver stem cells can differentiate into pancreas (18).

Recently, stem/progenitor cells have been isolated from both the ducts of the exocrine pancreas (19, 20), and the islets of Langerhans that make up the endocrine tissue of the pancreas (21-23). We have been characterizing a population of cells isolated from human pancreatic islets. These cells, nestin-positive islet-derived stem/progenitor cells (NIPs), initially express the protein nestin, a marker of neural stem cells, can be passaged extensively *in vitro*, and can be differentiated *in vitro* into islet-like clusters (ILCs) that produce islet hormones e.g. insulin and glucagon by their exposure to known differentiation agents such as the insulinotropic, neogenic hormone, glucagon-like peptide-1 (GLP-1) (21-23). Analyses of NIPs by flow cytometry show that they contain a substantial subpopulation of side population (SP) cells, similar to pluripotent hematopoietic SP cells (22).

One aspect of human NIPs that we are currently investigating is their potential efficacy to produce insulin in amounts sufficient to achieve glycemic control when transplanted into diabetic mice. Although we initiated the studies by transplanting human NIPs under the kidney capsules of immunosuppressed nude mice, we discovered that human NIPs successfully engraft when transplanted into immunocompetent C57BL/6 mice without a requirement for immunosuppression. Here we show that nestin-positive cells in the pancreas do not express either class I or class II major histocompatibility (MHC) antigens, describe the transplantation studies and some morphological characteristics of the xenografts, and demonstrate the development of mixed chimerism in the mice by the detection of donor human Y-chromosome and human-specific antigens. Sixty days after the intravenous administration of human NIPs to immunocompetent mice, we find by flow cytometry that 1.5% to 9.0% of the hematopoietic cells in spleen, bone marrow, and peripheral blood leucocytes (PBLs), express human HLA class I antigens. Further, these mice given NIPs by a single systemic intravenous injection develop focal regions of chimerism in multiple organs, including intestine, kidney, pancreas, heart, skeletal muscle and

brain, as detected by *in situ* hybridization using a human-specific probe to repetitive ALU sequences and by human ALU-specific polymerase chain reaction. We propose that human NIPs may induce a state of immune tolerance in immunocompetent mice such that the human tissue is recognized as self by the immune system of the mice. These findings suggest that when stem/progenitor cells derived from a human adult tissue (e.g., pancreatic islets) are transplanted into immunocompetent mice they appear to induce tissue tolerance, resist graft versus host disease, and differentiate into specific cellular phenotypes, defined by the expression of markers of epithelial tissue (mixed keratins) and mesenchymal tissue (vimentin) as well as the human-specific marker antigen for hematopoietic tissue (leukocyte common antigen, LCA, CD45). We suggest that our findings may hold promise for the use of these cells in future approaches to applications for the regeneration of degenerated tissues in human diseases.

Materials and Methods

Reagents

Basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) were obtained from Sigma (St. Louis, MO). Leukemia Inhibitory Factor (LIF) was obtained from Chemicon (Temecula, CA). The Y-chromosome DNA hybridization probe labeled with Spectrum Red was purchased from Vysis Inc. (Downers Grove, IL), and antibodies were from BD Pharmingen (Lexington, KY).

Mice

C57BL/6 mice, 8-10 weeks old, were obtained from Charles River Laboratories (Wilmington, MA) for use in the transplantation of human nestin-positive islet-derived stem/progenitor cells (NIPs) either under the kidney capsule or for systemic administration, by injection into the tail vein.

Isolation and culture of NIPs and karyotype analysis

Human islet tissue was obtained from the Juvenile Diabetes Research Foundation Center for Islet Transplantation, Harvard Medical School and the Diabetes Research Institute, University of Miami School of Medicine (Miami, FL). Nestin-positive islet-derived stem/progenitor cells (NIPs) were isolated and propagated as described previously (23). Briefly, islets were washed and cultured in RPMI 1640 medium containing 10% serum, 11.1 mM glucose, antibiotics, sodium pyruvate, β-mercaptoethanol, and growth factors. Within several days, nestin-positive cells grew out from islets. These cells were cloned and expanded in medium containing 20 ng/ml each of basic fibroblast growth factor and epidermal growth factor or, 10 ng/ml of hLIF (Chemicon International Inc., Temecula, CA) in the presence of serum. In certain instances the cells were maintained in the absence of serum. Chromosomal analysis and karyotyping were performed at the Dana Farber/HCC cytogenetics core laboratory, Brigham and Women's Hospital, Harvard Medical School.

Administration of human NIPs to immunocompetent mice

Between 10^5 and 10^6 NIP cells prepared from human male or female donor islets were transplanted under the kidney capsules of 26 female C57BL/6 mice without immunosuppression. The transplantation procedure has been described previously (24). The mice were sacrificed 15 to 60 days after the transplantations and the kidneys containing the NIP grafts were removed for histomorphological and biochemical analyses including fluorescence *in situ* hybridization (FISH) for the detection of human Y-chromosome, immunocytochemistry for detection of human-specific antigens, and detection of enhanced green fluorescent protein (EGFP). Four mice were each given a total of 10^5 to 10^6 NIPs intravenously via the tail vein, administered twice one month apart. Sixty days after the first i.v. injection of cells, the mice were sacrificed. Spleen, bone marrow, and peripheral blood were analyzed for the presence of chimerism by fluorescence-activated sorting of cells using the human marker

HLA – A, B, C. The pancreata, kidneys, livers, hearts, skeletal muscle, intestines, brains, and lungs were collected from the mice for *in situ* histohybridization using a DNA probe specific for the detection of human Y-chromosome, and human repetitive ALU DNA sequences as measures of tissue microchimerism.

Antibodies

The rabbit anti-human nestin was a gift from Dr. C. Messam (NINDS, NIH, Bethesda, MD). The mouse monoclonal antibody against human CD-45, HLA - A, B, C (Class 1 HLA marker), mixed keratins, and vimentin were purchased from BD Pharmingen (Lexington, KY). MHC I & II antibodies were from Serotec Inc. (Raleigh, NC).

Immunocytochemistry

Frozen kidneys with grafts were embedded in OCT compound and 5 µm tissue sections were prepared. Tissue sections were fixed in acetone, blocked with normal goat serum followed by avidin D and biotin blocking solution and then incubated overnight in mouse anti-human antibodies to CD45 (leukocyte common antigen, LCA) vimentin, and mixed keratins or in anti-rat antibodies to MHC I, MHC II, as described previously (21). Sections were rinsed with PBS and incubated with biotinylated goat anti-mouse serum. Sections were then immersed in DAB substrate solution and a red-brown precipitate was visualized. Immunostaining for insulin, glucagon, and PDX-1 of the subrenal capsular grafts was carried out on 5µm serial sections of frozen tissue. Antisera used were: guinea pig anti-insulin IgG and guinea pig anti-glucagon serum (Linco Research Inc., St. Charles, MO) and polyclonal antisera to human PDX-1 (IPF-1) (antiserum R253, (25)). Normal guinea pig IgG and normal rabbit serum (NRS) served as controls. Immunoreactivity was developed using both the glucose oxidase method, with NBT as the substrate, and the peroxidase method, with DAB as the substrate (Vector Laboratories, Burlingame, CA).

Human ALU sequence Histohybridization

Tissue sections were hybridized with a human ALU DNA probe kit following the recommendations of InnoGenex (San Ramon, CA). ALU sequences are repetitive DNA elements that are unique to primates. The human ALU DNA probe does not hybridize to mouse DNA at the specified hybridization conditions. Briefly, proteinase K reagent was added to cover the sections and was incubated at room temperature for 10-15 mins. Sections were then washed, re-fixed with 1% formalin and hybridized with the human ALU probe at 37°C overnight. Then, tissue sections were washed repeatedly with PBS-Tween 20 buffer and incubated with primary antiserum to the ALU probe followed by incubation with secondary antibody. Subsequently, sections were immersed in streptavidin-peroxidase substrate solution and the color was developed with an AEC (amino ethyl carbazole) chromogen(Sigma). Sections were counter-stained with hematoxylin and mounted in SuperMount for visual microscopic examination.

Polymerase Chain Reaction

PCR amplification of human Alu-sx and mouse c-mos sequences. DNA was extracted using the QIAamp DNA extraction kit (Qiagen, Valencia, CA). Alu-sx primers were ALU-FW: 5'-GGCGCGGTGGCTCACG-3' and ALU-REV: 5'-TTTTTTGAGACGGAGTCTCGCTC-3'. Primers for c-mos were MOS-FW: 5'-GAATTCAGATTTGTGCATACACAGTGACT-3', and MOS-REV: 5'-AACATTTTTCGGGAATAAAAGTTGAGT-3'. DNA amplification and primer selection were as previously described (26). PCR conditions were 94°C for 5 min followed by 40 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 60 sec, and final extension 72°C for 10 min. PCR products were resolved by electrophoresis on a 1.5% agarose gel, and transferred overnight to a cellulose filter. Southern blot hybridization was performed by autoradiographic detection with an ALU internal probe: 5'-CCACTTTGGGAGGCCGAGGCGGGTGGATCATGAGGTACAAGGTGAAACCC-

3'. Further confirmation of the presence of human sequences was achieved by cloning of the PCR fragments into the pCRII plasmid by TA cloning (Invitrogen Corporation, Carlsbad, CA) and direct sequencing (26).

Transfection of NIPs

Human NIPs maintained in long-term cultures containing serum and human LIF were transfected with a plasmid expressing the enhanced green fluorescent protein (EGFP) according to published protocols (GenePorter, Gene Therapy Systems, Inc., San Diego, CA). Cells were transfected sequentially twice within a period of four days to enhance the number of transfected cells. By this method approximately 20-30% of the cells expressed the green fluorescent protein.

Fluorescence In Situ Hybridization (FISH)

FISH was performed with a commercially available kit according to the manufacturer's recommendations (Vysis, Downers Grove, IL). Briefly, 5μm frozen sections on silanized slides were fixed with methanol and acetic acid, washed with PBS and 2x SSC at 73°C for 2 min. Then, sections were immersed in pepsin solution at 37°C for 5 mins, washed in 2x SSC at RT for 1 min and dehydrated for 1 min in 85% and 100% ethanol successively. The CEP Y-chromosome probe (orange fluorescence) mixture was denatured at 73°C, and applied onto the sections. Hybridization was performed at 37°C. Sixteen hours later, the slides were washed in 2x SSC for 1-4 min and mounted in a DAPI (blue fluorescence) containing solution to allow visualization of the nuclei.

Flow Cytometry Analysis

Peripheral blood leukocytes and bone marrow cells as well as splenocytes were isolated for flow cytometry (FCM) analysis (27). Cells were washed in Hanks Buffer containing 10% BSA, incubated with an Fc-receptor blocking solution and then incubated with PE-labeled mouse anti-human Class I MHC.

Cells obtained from animals that received tail vein injections of human NIPs, as well as control mice that did not receive NIPs, were analyzed by flow cytometry.

Results

Cytogenetic Analysis of NIPs

We have described previously the procedures for the isolation and culture of the NIPs (21-23). We have now examined the karyotype of the NIPs. Analysis of chromosome preparations of a NIP culture (clone Hu-6a, passage 06) revealed a 46, XY diploid cell line in two cells analyzed **(Figure 1)**. Analyses of three additional cells revealed 46 chromosomes.

Transplantation of NIPs under the kidney capsules of immunocompetent mice

NIP cultures were prepared from islets obtained from several different human male and female donors provided by islet procurement and transplantation centers. Human NIPs (10^5 to 10^6 cells) were transplanted under the kidney capsules of 26 immunocompetent female C57BL/6 mice over the course of 2 1/2 years. The mice were sacrificed from 15 to 60 days after the transplantations. Eight of the mice died within 24 h of causes related to the anesthesia and/or surgery. In 10/18 mice the NIPs successfully engrafted and grew into readily visible masses of tissue **(Figure 2A)**. The NIPs transplanted into 8/18 mice failed to engraft. We then set out to determine whether the grafts contained human tissue (xenografts). First, we transfected human NIPs with a plasmid expressing green fluorescent protein (EGFP) as shown in **Figure 2B**. These transfected NIP cultures were then transplanted under the kidney capsule of a mouse. Fifteen days later, the kidney was removed and under UV light the graft showed intense EGFP fluorescence **(Figure 2C)**. Later, the graft was excised, enzymatically dissociated and cultured for an additional 5d. Transfected NIPs present in explant cultures showed expression of EGFP **(Figure 2D)**.

Examination by H & E staining of the histology of the grafts showed circumscribed tissue with considerable pleomorphism without evidence of

invasion into the adjacent kidney parenchyma **(Figure 3A)**. In various grafts, and tissue sections of individual grafts, focal regions of tissue were seen that had the appearance of glandular epithelial tissue **(Figures 3B and 3C)**. The predominant tissue type appeared to consist of mesenchymal stromal-like tissue **(Figures 3D and 3E)**. These observations indicate that human NIPs engraft and survive for at least 60 days after transplantation under the kidney capsules of immunocompetent mice and appear to represent different tissue types. It should be noted that heretofore transplantation of human tissue xenografts into immunocompetent mice results in a complete rejection of the graft after 5 to 10 days (24, 27, 28). Therefore human NIPs may have a special property by which to induce the host (mouse) to recognize them as "self" and not to reject the xenograft. In this regard we show that the nestin-positive cells within rat pancreatic islets do not express either MHC I or MHC II antigens **(Figure 3F)**. Thus NIPs, as they reside in the islets, may be immunologically naive because they appear not to express either MHC I or MHC II antigens.

Examination of the xenograft tissues by immunohistochemistry using antibodies specific for human-specific leukocyte common antigen (LCA, CD45) a marker of hematopoietic tissues, keratin, an epithelial cell marker and vimentin, a mesenchymal marker, revealed clusters of immunopositive cells within the grafts concentrated along the border of the graft with the kidney **(Figure 4)**. The more peripherally-located tissue in the graft is probably of mouse origin, indicating a host vs. graft cellular proliferative response, but not typical of a vigorous graft rejection response. The reaction is more typical of a delayed sensitivity granulomatous response with fibrosis and scarring, rather than an acute rejection with lymphocytic infiltration.

We also examined the grafts for the expression of pancreatic endocrine tissue. In one mouse, a large graft examined 15 days after transplantation of human NIPs showed scattered islands of tissue that immunostained positive with antisera to the hormones insulin, glucagon, and the transcription factor PDX-1 **(Figure 4E)**. The expression of endocrine tissue was particularly prominent in

this mouse, but was also observed to a lesser extent in another transplanted mouse.

Evidence for tissue microchimerism originating from kidney NIP grafts

Because it seemed reasonable that the acceptance of the human xenografts by the mice was due to tissue tolerization by the establishment of chimerism we examined the various organs of the graft-bearing mice for evidence of microchimerism. Therefore immunohistochemical staining with antibodies to human LCA (CD45) was performed and revealed apparent microchimerism in the kidney parenchyma **(Figure 5)**. The establishment of microchimerism of donor cells in graft recipients is known to induce tolerance to donor tissue (29-35). In particular, the dendritic cells, special antigen presenting T cells, are known to induce tolerization (36, 37). To test for the presence of chimerism in organs of the female recipient mice other than the kidney, the FISH assay (fluorescence in situ hybridization) specific for the human male Y-chromosome (donor tissue), was performed. By semiquantitative assessment of human Y-chromosome positive nuclei present in 20-30 400x fields of tissue sections the prevalence of human cells was estimated to be 0.02 to 0.2%. This analysis indicates that microchimerism is widespread throughout many organs of the mouse including, kidney, pancreas, skeletal muscle, liver, and heart **(Figure 6)**. These findings in mice transplanted with human NIPs show that a preparation of human tissue islet–derived cells engraft when transplanted under the kidney capsules of fully immunocompetent mice and suggest that the mechanism for the acceptance of the xenografts is the establishment of tissue microchimerism.

Establishment of microchimerism by administration of human NIPs systemically to immunocompetent mice

To further investigate the potential capabilities of human islet-derived NIPs to successfully engraft in immunocompetent, nonimmunosuppressed mice we injected human NIPs (10^5 to 10^6 cells) into the tail veins of four immunocompetent C57BL/6 mice. The mice were sacrificed 60 days after the

systemic injection of the NIPs. The establishment of micro/mixed chimerism was examined by flow cytometry (FCM) of cells extracted from mouse bone marrow, spleen, and peripheral blood and by histohybridization of several organs of the mouse using a hybridization probe specific for the detection of human genome-specific ALU-repetitive DNA sequence elements, an assay for human tissue that became available during the course of these studies. ALU-repetitive DNA sequences are specific to the human genome and at the proper hybridization conditions the ALU hybridization probe does not cross-hybridize to mouse DNA.

The results of the FCM analyses using human-specific HLA – A, B, C monoclonal antibody showed substantial chimerism in bone marrow, spleen, and peripheral blood leukocytes (PBLs) of 0.71 to 9.2% **(Table 1 and Figure 7)**. The non-specific background of fluorescent cells was determined to be 0.6% for spleen and PBLs and 1.7% for bone marrow **(Figure 7).** By human ALU histohybridization chimerism was found in all tissues examined: small intestine, kidney, heart, skeletal muscle, liver, pancreas, and brain **(Figure 8)**. The distribution of ALU-positive cells in the tissues was very heterogeneous with limited focal regions of positive cells. Most of the tissue was negative, therefore, to obtain a semiquantitative overall assessment of the extent of human tissue microchimerism in the various organs of the mice, PCR products (semiquantitative) derived from the mouse organs were compared to that of a human DNA standard. It is estimated that the percentage of human cells in the organs (microchimerism) ranged overall from 0.15% (brain) to 0.005% (kidney, lung) although the distribution of human cells was heterogeneous in the mouse tissues **(Figure 9)**. These findings further demonstrate that human NIPs can take up residence in and thrive in various organs of immunocompetent mice for at least 60 days after their systemic administration into the mice.

Discussion

The results of these studies provide evidence that stem/progenitor cells, specifically stem/progenitor cells isolated from human pancreatic islets (NIPs), can cross a xenogenic barrier, as they engraft and grow when transplanted into

immunocompetent mice. The purpose of this initial study was not to extensively characterize the phenotypes of the NIPs in the various organs of the mice after their transplantation, but rather to demonstrate that a selected population of cells of human origin (NIPs) can engraft in immunocompetent mice without the requirement for immunosuppression. The findings presented herein demonstrate engraftment of a human tissue in immunocompetent mice. At this time it is not known whether such nestin-expressing cells derived from adult tissues can cross xenogenic barriers without host versus graft rejection, graft versus host disease, and oncogenicity, or whether our findings are unique to pancreatic islet-derived stem/progenitor cells.

However, Hori *et al.* (38) have recently reported the successful engraftment of nestin-positive, neurospheres derived from neural stem cells under the kidney capsules of immunocompetent allogeneically mismatched mice. These stem cells did not express MHC I or MHC II antigens and differentiate into glial and neurons concomitant with their extinction of nestin expression. A conclusion of their findings was that neural stem cells are immunoprotected. Perhaps, pancreatic islet-derived stem cells are similarly immunoprotected, as we report herein.

Although it was not the explicit intention of these initial studies to demonstrate differentiation of the NIPs into pancreatic endocrine tissue (Zulewski et al., 2001), we did find focal areas of tissue that costained with antisera to the endocrine markers insulin, glucagon, and PDX-1. Endocrine tissue staining was particularly pronounced in one graft **(Figure 4E)** but was also seen to a lesser extent in other grafts. We suggest that these findings represent an additional demonstration of principle that a subpopulation of cells exists within the NIPs that is capable of differentiating into pancreatic endocrine tissue (Zulewski et al., 2001).

The possibility has been raised that certain of the observations of the "transdifferentiation" of stem cells delivered into mice may be an artifact due to the fusion of marked stem cells with somatic cells, thus marking the somatic cell by introducing the marker into a pre-existing differentiated somatic cell (39, 40).

Although we cannot categorically exclude the possibility that extensive cell fusion has occurred in our studies, cell fusion appears to be a relatively rare event (41). The establishment of human Y-chromosome-positive cells originating from the human NIPs transplanted under the kidney capsules, was widespread in all of the organs examined showing one to three Y-chromosome positive cells per 400x magnified field of view. It should be noted that the efficiency of detecting the presence of the human Y-chromosome in a nucleus in a 5 μm tissue section is about 20-50% because of the residence of the Y-chromosome in a single locus of the nuclear chromatin. Thus, the extent of human Y-chromosome chimerism is estimated to be 0.20-0.02% of all cells in the various organs examined. Furthermore when the human NIPs were delivered to the mice by systemic intravenous injection, the extent of tissue chimerism was substantial when analyzed by histohybridization using a probe to human-specific ALU repeat sequences. As such, every human chimeric cell will be detected in the nuclei of a 5 μm tissue section. Because of the large DNA target presented by the 3-6 x 10^5 copies of the human ALU sequence the histohybridization conditions used to detect these sequences was stringent. The density of human ALU-positive cells in the tissue sections of the various organs is difficult to assess because ALU-positive cells were not evenly distributed throughout the sections, but rather were present at relatively high density in scattered focal regions and at low density, or not detectable at all, in other regions of the tissue sections. Some focal regions of chimerism in the brain, heart, and kidney were in the range of 20 to 5% by counting ALU-positive and ALU-negative cell nuclei per selected field in sections of the various organs. By semiquantitative analysis of the tissues, which includes the entire tissue, the PCR prevalence of chimerism appears to be between 0.16 to 0.005%, with the brain being the highest.

In addition to our demonstration of successful xenoengraftment without immunosuppression, we provide evidence that the lack of host versus graft rejection appears to result from the establishment of tolerizing mixed chimerism of the transplanted human tissues in mouse organs. Over 30 years ago it was recognized that transplanted organs (allografts) in human recipients become

genetic chimeras (34, 42). In 1992 Starzl et al. (31) showed that not only is the donor organ populated by host recipient cells, but that the host recipient organs became populated by donor cells. This mutual cross-population of transplanted donor cells and recipient organs was termed microchimerism. This microchimerism was proposed as a mechanism to explain the induction of immune tolerance (host vs. graft) and the avoidance of graft vs. host disease; owing to the capacity of passenger hematopoietic cells within the donor organ to convert to dendritic T-cells. Dendrite cells are specialized highly efficient antigen-presenting cells that function via the thymus to "re-educate" host tissue-reactive T-cells to recognize the donor allograft as self. It was postulated that the mixed chimeric state results from a two-way interaction between donor and recipient leukocytes vying for dominance, similar to a two-way mixed lymphocyte reaction (31, 34). The balance between the recipient immune system and the donor leukocytes that determines whether the outcome is graft rejection (recipient leukocytes dominate), graft vs. host disease (donor leukocytes dominate), or a mutually acceptable tolerant state develops between the transplant and host acceptance. It has been suggested that the donor-tolerizing dendritic cells must originate from a population of "stem" cells present in the donor organ to explain the existence of foreign tissue tolerance by the host for 30 years or more (33). Some organ transplant recipients have self-discontinued immunosuppression therapy and the grafts are maintained functionally intact, due to the induction of immune tissue tolerance via the establishment of microchimerism, macrochimerism, or mixed chimerism (30).

Much effort has been made in the co-administration of donor blood transfusions or bone marrow transplants concomitant with organ transplants, but their relevance for graft acceptance is still a matter of debate (29, 32, 35). The potentially immunomodulating and tolerizing mechanisms of donor-specific transfusions and donor bone marrow transplants appear to be similar and include induction of anergy, stimulation of anti-HLA antibodies, provision of soluble antigen, suppressor cell and/or veto cell activities, clonal deletion, regulation of all surface molecules, regulation of cytokines, promotion of microchimerism, or

any combination of these circumstances (reviewed in and summarized from (43)). A conjecture is that stem cells, resident in and released from the donor transplants, are responsible for tolerization, perhaps in addition to passenger immunotolerant-prone dendritic T-antigen-presenting T-cells (33, 36, 37, 44). We suggest that our findings of xenoengraftment of the human NIPs transplanted under the kidney capsules, or by intravenous injection into the systemic circulation, of non-immunosuppressed mice (the cells grow and are not rejected by the usual criteria of a rapid, vigorous rejection of a xenograft) manifest a property of stem-like progenitor cells as they appear to induce immune tolerance and thereby resist host vs. graft rejection. Somehow the NIPs must re-educate the host immune system to recognize the donor stem/progenitor cells as self. This may occur by mechanisms of either microchimerism (peripheral), macrochimerism (central), or both (mixed chimerism).

The possible efficacy of NIPs to restore injured tissue when used as regenerative medicine remains unknown. These proof of principle studies as yet need to be done. Because of the finding of tolerization and the establishment of tissue chimerism after the administration of human tissue-derived cells to immunocompetent mice it is tempting to speculate that NIPs or NIP-like cells may conceivably be useful for the therapeutic induction of tissue tolerance. One might conjecture that such induction of tolerance could be a component of the protocol in certain organ transplantation procedures, and thereby eliminate the need for immunosuppression. For example, when a voluntary organ (e.g. kidney) donor is identified, tissue could be obtained from the donor, NIP or NIP-like cells could be isolated from the tissue and expanded *in vitro*. The cells could then be administered to the potential recipient to tolerize the recipient to the donor organ. The induction of a state of tolerance to the donor tissue in the recipient could be determined by taking a skin biopsy from the donor and determining successful test engraftment in the potential recipient.

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Figure Legends

Figure 1: Karyotype of human NIPs. The NIPs were passaged in continuous culture for five months. The NIP culture was derived from pancreatic islets obtained from a male donor pancreas. The karyotype is that of a diploid (euploid) 46, XY male.

Figure 2: Engraftment of human NIPs in immunocompetent mice.

A. Mouse kidney removed 35 days after transplantation of human NIPs under the kidney capsule. The mouse was a female C57BL/6 without immunosuppression. The whitish mass of tissue at the top of the kidney (arrow) is the expanded NIP graft, contrasting with the kidney tissue. The engraftment of NIPs has been achieved in 18/26 of non-immunosuppressed mice so transplanted and examined from 15 to 60 days after the xenograft. **B.** A human NIP transfected with a plasmid expressing enhanced green fluorescent protein (EGFP) viewed under UV light. The cells expressing EGFP were transplanted under the kidney capsule of an immunocompetent mouse without immunosuppression. **C.** Gross morphology of the human NIP graft expressing EGFP in a kidney removed 15 days after transplantation of the NIPs. The graft (G) and kidney (K) were viewed under UV light. **D.** EGFP-positive cells present in explant cultures prepared from the graft shown in C (5d culture).

Figure 3: Histomorphology of a human NIP graft in an immunocompetent mouse.

A. Tissue sections (H and E stained) of a C57BL/6 non-immunosuppressed mouse kidney and human NIP graft 35 days after transplantation of human NIPs under the kidney capsule (magnification 100x). K, mouse kidney; G, human NIP graft. Note that the transplanted human NIP cells grow and are not rejected by the mouse recipient of the graft. **B.** The graft contains localized regions of glandular-like tissue (liver). **C.** 1000x magnification of glandular-like liver tissue in graft. **D.** Mesenchymal-stromal-like tissue in the graft . **E.** 400x magnification of the stromal-like tissue in the graft. **F.** Nestin-positive cells in the pancreas do not

express either Class I or Class II MHC antigens. Dual immunostaining (green) of a rat pancreas with antisera to MHC Class I antigen (left) and MHC Class II antigen (right). Nestin-positive cells are immunostained in red. The absence of yellow cells which would indicate that a cell co-expresses nestin and MHC antigens shows that NIPs do not express either MHC Class I or Class II antigens. Dashed lines denote boundaries of pancreatic islets.

Figure 4: Immunohistochemical analyses of human NIP grafts in immunocompetent mice. A. Immunostaining for human-specific leukocyte antigen (LCA), CD45, in a section of mouse kidney transplanted (kidney capsule) with human NIPs examined 35 days after the transplant. The brown-colored cells (shown in the figure as grayish-black) are the cells in the xenograft that express human LCA in the recipient mouse. K=kidney; G=graft. (100x) **B.** LCA immunostaining of the graft shown at higher magnification. (400x) **C.** Keratin immunostaining (400x). **D.** Vimentin immunostaining (400x). **E.** Detection of pancreatic endocrine hormones insulin and glucagon, and pancreas- and duodenal-specific homeobox transcription factor, PDX-1 in serial sections of expanded grafts of human NIPs 15 days after transplantation under the kidney capsules of immunocompetent C57BL/6 mice. Islands of pancreatic endocrine-like tissue are found scattered about in the graft which grew to about 50% the volume of the kidney without evidence of invasion or oncogenicity. The upper two rows of panels show two representative fields of the grafts (100x). GP-IgG and NRS are the control antisera for insulin and glucagon/PDX-1 respectively (lower two panels). Immunostaining done by glucose oxidase method (shown in the figure as grayish-black). Immunocytochemistry was also done by DAB (Vectastain ABC) with similar results. Asterisk denotes a burnout defect artifact in the CCD camera.

Figure 5. Evidence for the establishment of microchimerism of human tissue in the kidney parenchyma of an immunocompetent mouse given a subrenal capsular graft of NIPs 35 days before. **Left panel:** Renal tubules within a section

of kidney immunostained with an antibody specific for human leukocyte common antigen (LCA, CD45). Magnification 400x. **Right panel:** Low power micrograph (100x) of the NIP engraftment kidney as it appears 35 days after the NIP transplant under the kidney capsule. Immunostained with antibody to LCA/CD45. G=graft, K=kidney.

Figure 6. Fluorescence in situ histohybridization (FISH) of human Y-chromosome in tissues of a female mouse 45 days after the transplantation of 10^6 human male NIPs under the kidney capsule of an immunocompetent C57BL/6 mouse. A. Kidney **B.** Liver **C.** Skeletal muscle. **D.** Exocrine pancreas **E.** Endocrine pancreas (islet) 400x magnification. One to three human Y-chromosome positive cells were observed in representative fields.

Figure 7. Flow cytometry using fluorescence-activated cell sorting of cells obtained from immunocompetent mice 60 days after systemic administration of human NIPs; the tissues are: A. bone marrow (BM), **B.** spleen (SP), and **C.** peripheral blood leukocytes (PBLs). The fluorescent marking probe was a monoclonal antibody to HLA antigens A, B, C. The FCM data shown correspond to mouse #2 in Table 1.

Figure 8: Histohybridization to detect human-specific ALU-repetitive sequences in tissues of female immunocompetent mice 60 days after a single intravenous injection of 10^6 human NIPs.
A. The NIPs in culture used for the intravenous systemic injections in mice. **B.** small intestine, **C.** kidney, **D.** heart, **E.** skeletal muscle, **F.** liver, **G.** pancreas, **H.** brain. The brown stained nuclei are positive for the presence of human-specific ALU sequences. The blue nuclei counterstained with hematoxylin are negative for human ALU sequences. Magnification is 400x. B is mouse #1 in Table 1. C & D are from mouse #2 in Table 1.

Fig 9. Detection of human ALU-repetitive sequences and mouse c-mos in different organs of transplanted mice by PCR. A. Ethidium bromide staining of an SDS-PAGE gel. Lane 1, 100 bp molecular weight marker; lane 2, positive control (60 pg human DNA); lane 3, negative control (240 pg mouse DNA); lanes 5-11, 20 ng of DNA from different organs of a transplanted animal. Lane 5, liver; lane 6, brain; lane 7, muscle; lane 8, kidney; lane 9, intestine; lane 10, lung; and lane 11, heart. **B.** Southern blot of gel shown in panel A using a labeled ALU sequence probe. **C.** Semiquantitatively densitometric analysis of products detected in the Southern blot shown in B.

pilot study - Exhibit D

Transplantation of embryonic porcine mesencephalic tissue in patients with PD

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Article abstract—*Objective:* To assess the safety and the effect on standardized clinical rating measures of transplanted embryonic porcine ventral mesencephalic (VM) tissue in advanced PD. *Methods:* Twelve patients with idiopathic PD underwent unilateral implantation of embryonic porcine VM tissue; six received cyclosporine immunosuppression and six received tissue treated with a monoclonal antibody directed against major histocompatibility complex class I. Patients were followed for 12 months and assessed by clinical examination, MRI, and ^{18}F-levodopa PET. Porcine endogenous retrovirus testing was conducted by PCR-based method on peripheral blood mononuclear cells. *Results:* Cell implantation occurred without serious adverse events in all patients. Cultures were negative for bacterial and unknown viral contamination. No porcine endogenous retrovirus DNA sequences were found. MRI demonstrated cannula tracts within the putamen and caudate, with minimal or no edema and no mass effect at the transplant sites. In the medication-off state, total Unified Parkinson's Disease Rating Scale scores improved 19% ($p = 0.01$). Three patients improved over 30%. There were two patients with improved gait. ^{18}F-levodopa PET failed to show changes on the transplanted side. *Conclusions:* Unilateral transplantation of porcine embryonic VM cells into PD patients was well tolerated with no evidence of transmission of porcine endogenous retrovirus. Changes in standardized clinical PD rating measures were variable, similar to the results of the first trials of unilateral human embryonic allografts that transplanted small amounts of tissue. **Key words:** PD—Xenotransplant—Porcine retrovirus—Embryonic cell transplant—Monoclonal antibody.

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Pharmacologic therapy in PD is often effective in the early stages of the illness, but after 5 to 10 years symptom control is less successful owing to dyskinesias and other response fluctuations. Cellular replacement therapies for PD were considered following the amelioration of motor deficits in dopamine-depleted rodents after transplantation of embryonic dopaminergic cells.[1,2] The first human embryonic ventral mesencephalic (VM) transplants in idiopathic PD were reported in 1988.[3] Since the initial reports, embryonic human VM tissue has been stereotactically implanted in over 250 patients with idiopathic PD and neurotoxin-induced parkinsonism.[4-9] The published outcomes of these procedures demonstrate that transplanted embryonic human dopaminergic neurons survive, and ameliorate many of the motor and medication-induced symptoms of advanced PD; sustained clinical improvement has been observed.[4-9]

The limited availability of human embryonic tissue and ethical concerns[10] limit widespread clinical application to PD patients. These obstacles have prompted consideration of alternative tissue sources, including the use of xenogeneic donor tissue. The pig has been considered the most desirable source species of whole organs.[11] In addition to yielding a large litter size, pigs provide optimally staged embryonic porcine tissue that is physiologically similar to human dopaminergic neurons. This tissue can be relatively easily collected, and the brain region containing the developing VM can be reliably dissected. Animals can be raised under controlled quarantined conditions and screened for bacterial and viral diseases.

Porcine VM xenografts survive and correct motor deficits in a PD animal model.[12-14] Therefore, a pilot

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Table 1 Baseline patient characteristics

Characteristic	Value
Sex, male/female	9/3
Age, y, mean ± SD	60.8 ± 6.5
Disease duration, y, mean ± SD	14.0 ± 5.9
Hoehn-Yahr stage*	
"Off" period, median	3.4
"On" period, median	2.3
Medication, mg, mean ± SD	
Levodopa equivalents,† n = 12	908 ± 615
Levodopa + PDCI, n = 12	734 ± 543
Bromocriptine, n = 3	25.8 ± 11.8

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* Ten evaluable patients.

† Levodopa equivalent = (1.0 × levodopa) + (0.75 × levodopa in Sinemet CR) + (10 × bromocriptine) + (100 × pergolide); all doses in milligrams.

PDCI = peripheral decarboxylase inhibitor.

study was initiated to assess the safety and effect on standardized PD clinical rating instruments of unilateral implantation of embryonic porcine VM cells into the striatum of patients with advanced PD. In this study we report 1 year follow-up data of the first implantation of xenogeneic neural tissue into humans. As transmission of infectious pathogens from the animal source tissue to the human recipient is an inherent risk of xenotransplantation,[15] an assay was developed by the authors to test patients for the presence of a recently reported porcine retrovirus.[16]

Methods. *Patients.* Between April 1995 and October 1996, 12 patients with idiopathic PD underwent unilateral implantation of embryonic porcine VM tissue (table 1). All patients had advanced PD, were levodopa-responsive, experienced "off" periods or dyskinesias, and exhibited at least two of the following symptoms: bradykinesia, rigidity, tremor, or postural instability (one symptom was either bradykinesia or tremor). Most patients had been treated with dopaminergic agonists (see table 1). Patients were excluded if they exhibited:

1) evidence of secondary parkinsonism or Parkinson's plus syndromes;
2) a history of cerebral vascular disease or seizures;
3) prior intracranial surgery;
4) an MRI consistent with cortical atrophy or significant intracranial lesions;
5) any medical illness that would interfere with surgery or the ability to take cyclosporine immunosuppression;
6) significant cognitive impairment (Mini-Mental State Examination score less than 23) or clinical depression (Hamilton Depression score greater than 19).

Institutional review board and informed consent. The experimental protocol was reviewed and approved by the institutional review boards of Boston Medical Center and the Lahey Medical Center. The Food and Drug Administration reviewed the protocol and consent. Informed consent was obtained from patients in the study. No patient received money or was paid for inclusion into the study.

Study design. Clinical evaluations were conducted according to many of the guidelines described in the Core Assessment Program for Intracerebral Transplantation (CAPIT),[17] which included assessments during "on" and "off" periods using the Unified Parkinson's Disease Rating Scale version 3.0,[18] Hoehn and Yahr score, and timed motor tests (wrist pronation supination, finger dexterity, arm movements, and stand-walk-sit test). The clinical assessments in the "off" state were made when the patient's antiparkinson medication had been withheld for at least 12 hours. This core evaluation was conducted preoperatively on two occasions in eight patients; the first four patients received one preoperative core evaluation. MRI of the brain was performed preoperatively and postoperatively within 1 week and 6 months after surgery. The integrity of the striatal dopaminergic system was assessed preoperatively and postoperatively at 6 and 12 months by PET scans using ^{18}F-fluorodopa as the tracer.

Six patients were transplanted with embryonic porcine neural cells in combination with standard immunosuppression (cyclosporine). A preoperative loading dose of cyclosporine (9 to 15 mg/kg) was given 12 hours before transplantation. Postoperative daily doses of cyclosporine were titrated to achieve a trough whole blood level of 100 to 150 ng/mL. The other six patients were transplanted with embryonic porcine neural cells that had been treated with an F(ab')$_2$ fragment of a monoclonal antibody directed against major histocompatibility complex class I (MHC I). This immunomodulatory technique has been shown in animals to permit xenograft survival without systemic immunosuppression.[19,20]

Preparation of embryonic porcine VM cells. The identification of donor animals and the preparation of embryonic porcine VM cells was carried out under rigorous manufacturing controls and in accordance with many of the recommendations provided in the Draft Public Health Service Guideline on Infectious Disease Issues in Xenotransplantation.[21] Donor animals from a prescreened Yorkshire herd were isolated, screened by serology for evidence of exposure to certain pathogens, tested for parasites, and tested for mycobacterium tuberculosis by skin test. The initial serology screen included testing for 12 pathogens known to be present in the United States and that are zoonotic, are neurotropic, or cross the placental barrier. In addition, animals were wormed and vaccinated for erysipelas, leptospira, and parvovirus. Selected donors that passed the initial screening process were moved to a designated isolation room, maintained according to documented husbandry procedures, tested for parasites and bloodborne viral pathogens, and inseminated with semen obtained from a prescreened boar or a prescreened source of artificial semen.

Harvest of embryonic tissue was performed in a dedicated clean room that is maintained under positive pressure, using quality control procedures and criteria for lot release. Embryonic mesencephalic cell suspensions were prepared by dissection of the VM brain region from embryonic day-25 to day-28 porcine fetuses. Time-mated, ultrasound-confirmed pregnant Yorkshire pigs were eutha-

nized according to the standard veterinary procedures at Tufts University School of Veterinary Medicine and uteri were obtained under aseptic conditions. The VM regions from the fetal brains were dissected under microscopic guidance, pooled, incubated, and trypsinized to prepare a cell suspension for transplantation. The cell suspension was prepared at 50,000 cells/μL of defined medium (glucose and normal saline). Anti-MHC I monoclonal antibody-treated porcine embryonic cells were prepared by incubating the cell suspension with a mouse-derived anti-MHC I monoclonal antibody $F(ab')_2$ fragment (10 μg/mL).[19] Before surgical implantation, it was determined that cell suspensions used for transplantation had greater than 70% viability (by an acridine orange/ethidium bromide staining) and were negative for bacteria by Gram stain. Aliquots of the cell suspension were cultured for identification of dopaminergic neurons (by staining with an antibody to tyrosine hydroxylase), bacterial contamination, and presence of viruses (detected by cytopathic effect with indicator cell lines); results from these studies were available after the implantation procedure.

Surgical implantation of embryonic porcine VM cells. Eleven patients underwent the following surgical procedure. Using local anesthesia and MRI- and CT-guided stereotactic neurosurgical technique (CRW system, Radionics, Burlington, MA), 80-μL volumes of cell suspensions were deposited unilaterally in the striatum along three separate tracks, each 5 mm in length: one track in the head of caudate, one track in the mid putamen, and one track in the posterior putamen. Four million cells in 240 μL were deposited along each track for a total of 12 million cells per striatum. The second patient to undergo surgery received a total of 200 μL of cell suspension infused into three putamen sites and 40 μL of cell suspension infused into one caudate site. Cells were delivered at a rate of approximately 4 μL/minute. At the end of the infusion, the cannula was left in place for 1 minute, and then removed. The cell infusion cannula (Radionics) had an outer diameter of 0.7 mm and an inner diameter of 0.5 mm. The location of infusion sites was confirmed by MRI 1 week postoperatively. During the surgical procedure patients received local anesthetic and, if necessary, mild sedation. A broad-spectrum antibiotic (cefazolin, 1 g) was administered pre- and postoperatively.

Safety assessment. Standard adverse event reporting was conducted at regular intervals. Patients receiving cyclosporine therapy were evaluated approximately every month in order to adjust cyclosporine dose. During each safety evaluation, routine blood chemistry, hematology, and urinalysis were performed.

Porcine endogenous retrovirus testing. Peripheral blood mononuclear cells (PBMCs) from 11 patients, which had been obtained preoperatively and periodically postoperatively, were assayed for the presence of porcine endogenous retrovirus (PERV) DNA sequences using a PCR-based method. Frozen patient PBMCs were thawed and DNA was obtained using phenol/chloroform/isoamyl alcohol extraction and alcohol precipitation. Ten microliters of each sample (estimated to contain DNA from approximately 1×10^5 cells) was amplified in a PCR amplification using primers specific to the protease region of PERV.[16] Conditions for the thermal cycling were: 10 minutes at 22 °C ± 2 °C followed by 2 minutes at 95 °C ± 2 °C; 35 amplification cycles with a final extension cycle. Amplification products were separated by electrophoresis on an agarose gel and transferred to nylon membranes. Specific products were detected by Southern blot hybridization using as a probe a fluorescein-dUTP–end-labeled oligonucleotide complementary to the amplified PERV sequence and a chemiluminescent detection system (Amersham; Arlington Heights, IL). Control reactions using PERV primers included DNA from the following sources: porcine PBMCs; nontransplanted patients' PBMCs; porcine cell lines known to contain PERV (PK-15)[16]; and patient PBMCs spiked with porcine buffy coat DNA. To confirm that patient DNA was a suitable template for PCR amplification, human-specific β-globin primers were used to amplify patient DNA samples. This assay was capable of detecting PERV DNA sequences at a sensitivity of 0.1 cell.

PET imaging with 6-[^{18}F]fluoro-L-DOPA. PET studies were performed with a PC-4096 scanner (Scandtronix AB, Uppsala, Sweden). Patients fasted overnight and all antiparkinson medications were withheld for 12 hours before PET imaging. Carbidopa (200 mg) was administered orally 1 hour before tracer injection to inhibit dopa decarboxylase. Subjects were positioned in the scanner in individually fabricated head holders (Tru Scan Image, Annapolis, MD) with laser alignment. The gantry angle was adjusted to be parallel to the orbitomeatal line. [^{18}F]fluorodopa (4 to 10 mCi prepared as described previously[22]; radiochemical purity >95%, specific activity 400 mCi/mmole) was injected IV as a bolus at the start of imaging. Images were acquired over a period of 120 minutes in a series of 24 frames whose durations progressively increased from 0.5 to 10 minutes. To enhance visualization of the striatum, frames 10 to 24 were summed. Using this summed image and guidance from MRI, the putamen, caudate, and occipital cortex were outlined to place regions of interest (ROI). The putamen region was subdivided into anterior and posterior segments. The ROIs were used to construct concentration history curves for extracting PET data in the corresponding locations in frames 1 to 24. The striatal to occipital ratio (SOR) was computed from the data taken at 60 to 90 minutes. The same set of ROIs was used to analyze each scan for a single subject. When necessary, locations were adjusted to compensate for repositioning.

Data analysis. Pre- and postoperative clinical data were obtained for 10 patients (four in the cyclosporine group, six in the antibody group). Group data for these 10 patients through the 12-month postoperative visit are reported. Comparisons between the resulting baseline evaluation and the postoperative evaluation were performed with a paired Student's *t*-test. All tests were performed at the 5% significance level. Unless otherwise specified, data are presented as mean ± SD. When two preoperative assessments were performed, the mean of these values was

calculated and used as the baseline value. For two patients in the cyclosporine group (the first two patients to undergo surgery), pre- or postoperative data were not obtained according to CAPIT guidelines.[17] Data from the first patient are reported separately. No 12-month data are available for the second patient as this patient died 7.5 months after surgery.

Results. *Safety.* Adverse events. The neurosurgical procedure was well tolerated and all patients were discharged from the hospital within 72 hours. No serious adverse events were judged by the investigators to be definitely related to the cell implantation or surgical procedure. Patient 8 fell off a bicycle 3 months after surgery and had a small subdural hematoma (ipsilateral to the surgical site), which was associated with transient contralateral paresthesias. Patient 12 was admitted to a hospital 3 months after surgery for adjustment of antiparkinson medication. Six months after surgery, Patient 2 had an episode of confusion related to antiparkinson and pain medication. This patient died from acute pulmonary emboli 7.5 months after surgery. Twelve months after transplantation, Patient 3 experienced transient upper extremity shaking contralateral to the surgery; this event resolved without sequelae or recurrence. The etiology of the event was undetermined. Patient 10 had a fall resulting in a fractured leg 12 months after surgery.

Postoperative MRIs performed before discharge showed cannula tracts within the putamen and caudate, with minimal or no edema. Postoperative MRIs performed 6 and 12 months after surgery showed no mass effect or contrast enhancement at the transplant sites.

Bacteriologic and viral testing of implanted porcine tissue. Postoperative cultures of aliquots of the implanted tissue were negative for bacterial and unknown viral contamination. The presence of PERV nucleic acid sequences was examined in postsurgical PBMCs obtained at the following time points after surgery (number of patients at each time point in parentheses): 6 months (4 patients), 9 months (2 patients), 12 months (2 patients), 15 months (1 patient), 18 months (1 patient), and 24 months (1 patient). PERV DNA sequences were not detected in the PBMCs from these 11 transplant recipients.

Neuropathologic analysis of Patient 2. Detailed description of the neuropathologic analysis of Patient 2 has been previously reported.[23] Three cannula tracks were found in the putamen. Using histologic markers for porcine genomic sequences and porcine neurofilaments, survival of porcine neurons and glia was observed. Using immunohistochemistry to tyrosine hydroxylase, approximately 650 dopaminergic neurons were detected within the grafts. There was minimal lymphocytic infiltration and glial reaction at the graft sites, and no evidence of active rejection.

PD clinical rating assessments. At 12 months after implantation, the total UPDRS score in the "off"-period state decreased (improved) approximately 19% in the group of 10 evaluable patients ($p = 0.01$; table 2). The activities of daily living (ADL) subscore and the derived postural instability gait disorder (PIGD) score[24] improved in the "off"-period state, but the UPDRS motor and the Hoehn and Yahr subscores did not. The "on"-period total UPDRS, ADL, Hoehn and Yahr, and PIGD scores did not improve.

***Table 2** Mean ± SD "off"-period and "on"-period scores before and 12 months after unilateral embryonic porcine ventral mesencephalic cell implantation (for 10 evaluable patients)*

Scores	Baseline	12 Months	*p* Value
"Off"-period			
Total UPDRS	83.7 ± 23.0	66.8 ± 21.0	0.010
ADL*	27.1 ± 6.8	20.4 ± 7.9	0.006
Motor*	46.6 ± 16.4	39.5 ± 14.0	0.123
Hoehn & Yahr	3.7 ± 0.8	3.4 ± 1.3	0.148
PIGD†	12.2 ± 4.5	8.6 ± 5.4	0.003
"On"-period			
Total UPDRS	32.0 ± 8.9	25.9 ± 16.8	0.137
ADL*	8.8 ± 3.8	7.1 ± 6.2	0.312
Motor*	13.1 ± 6.0	11.9 ± 10.1	0.686
Hoehn and Yahr	2.2 ± 0.5	2.1 ± 0.4	0.065
PIGD†	4.0 ± 1.9	3.2 ± 3.0	0.418
Subscores of UPDRS			
Mentation	1.5 ± 2.0	1.4 ± 2.0	0.811
Complications	8.5 ± 2.0	5.5 ± 2.3	0.002
DOPA equivalents‡	962 ± 647	923 ± 516	0.624

For supplementary data, please visit our Web site (www.neurology.org) and access the data through the Table of Contents page of the March 14 issue.

* Subscores of Unified Parkinson's Disease Rating Scale (UPDRS).

† Postural Instability Gait Disorder Score; the numerical sum of questions number 13 (falling), 14 (freezing when walking), 15 (walking), 29 (gait), and 30 (postural stability), from UPDRS.[18]

‡ Levodopa equivalent = (1.0 levodopa) + (0.75 × levodopa in Sinemet CR) + (10 × bromocriptine) + (100 × pergolide); all doses in milligrams.

ADL = activities of daily living.

The complication subscore improved significantly, but the mentation subscore did not.

Compared with baseline scores, the magnitude of improvement in the total UPDRS (table 3) "off"-period scores at 12 months after surgery was approximately the same in the cyclosporine group (19.6%; from 66.9 ± 13.5 to 53.4 ± 18.0, $p = 0.2$) and the MHC I antibody group (19.0%; from 94.9 ± 21.5 to 75.7 ± 19.0, $p = 0.04$). The improvement in the ADL "off"-period score appeared greater in the cyclosporine group (from 23.5 ± 2.0 to 13.8 ± 5.2, $p = 0.02$) than in the MHC I antibody group (from 29.5 ± 7.9 to 24.8 ± 6.0, $p = 0.14$). Improvement in the total UPDRS "off"-period score was observed at the first postoperative evaluation at 3 months (figure). The magnitude of the improvement remained approximately the same throughout the 12-month assessment (see the figure).

There was a wide range of improvement of total UPDRS "off"-period scores among patients (see table 3). Patients 3, 7, and 11 improved over 30% (34, 45, and 51%). Patients 4 and 5 showed a mild improvement (25 and 19%). For five patients (Patients 6, 8, 9, 10, and 12), the change in UPDRS score was 11% or less (see table 3).

From baseline to 12 months, there was no change in UPDRS "off"-period motor subscore in the cyclosporine

Table 3 Mean ± SD total UPDRS "off"-period scores in cyclosporine and anti-MHC I antibody–treated patients before and 12 months after porcine cell implantation

Patient	Baseline	12 Months	% Change
Cyclosporine			
3	54.5	36.0	−33.9
7	75.3	41.5	−44.8
8	56.3	61.0	8.5
9	81.5	75.0	−8.0
Total	66.9 ± 13.5	53.4 ± 18.0	−19.6 ± 24.3
Anti-MHC I antibody immunomodulation			
4	128.5	96.5	−24.9
5	104.3	84.5	−18.9
6	76.5	69.5	−9.2
10	68.5	69.5	1.4
11	90.0	44.0	−51.1
12	101.5	90.0	−11.3
Total	94.9 ± 21.5	75.7 ± 19.0	−19.0 ± 18.1

UPDRS = Unified Parkinson's Disease Rating Scale; MHC = major histocompatibility complex.

group (from 35.0 ± 14.5 to 34.1 ± 14.3, $p = 0.90$) or anti-MHC I antibody group (from 54.5 ± 13.2 to 43.0 ± 13.8, $p = 0.08$). Nonetheless, the two patients who demonstrated the largest improvement in total UPDRS "off"-period scores between baseline and 12 months also demonstrated a large improvement in the "off"-period motor subscores (Patient 7, from 44.3 to 25.5; Patient 11, from 54.0 to 12.5) (for supplementary data, please visit our Web site at www.neurology.org and access the data through the Table of Contents page of the March 14 issue). Moreover, upper extremity timed motor scores for the group were faster bilaterally in the "off" period and "on" period, but ipsilateral finger tapping did not change.

Changes in UPDRS and timed motor scores do not appear to be due to changes in medication, as the mean levodopa equivalents (levodopa equivalent [mg] = [1.0 × levodopa] + [0.75 × levodopa dose in Sinemet CR] + [10 × bromocriptine] + [100 × pergolide]; all doses in milligrams) in the 10 evaluable patients did not change between baseline and 12 months (for supplementary data, please visit our Web site at www.neurology.org and access the data through the Table of Contents page of the March 14 issue).

Even though the postsurgical improvement in CAPIT scoring could not be quantified for baseline in Patient 1, this patient exhibited clinical improvement after transplantation (the preoperative "off"-period scores could not be obtained according to the CAPIT protocol until 4.5 months postoperatively; preoperatively, when medications were withheld for more than 12 hours, the patient could not be transported to the clinical site owing to severe immobility). His severe "off"-period akinesia and dyskinesias were much less by 12 months, leading to a meaningful clinical improvement. Between 4.5 months (the first evaluable time point) and 12 months, his "off"-period total UPDRS scores improved from 117.5 to 68.5, and motor subscore improved from 79.5 to 46.5.

The patients with the largest improvements in total UPDRS "off"-period scores (Patients 1, 7, and 11) demonstrated improvement in timed motor tests (for supplementary data, please visit our Web site at www.neurology.org and access the data through the Table of Contents page of the March 14 issue). These improvements were bilateral, although in Patient 1 there was a greater improvement, compared with baseline, between 4.5 months and 12 months in arm speed contralateral (from 13.0 to 9.1 seconds) compared to ipsilateral (from 9.0 to 8.2 seconds) to the surgery. Before transplantation, 2 of the 12 patients (Patients 1 and 11) exhibited "off"-state gait freezing and were unable to perform the stand-walk-sit test in the "off"-period. At 6 months (Patient 1) and 3 months (Patient 11) after surgery both patients were able to walk in the "off"-state, and walking speed improved progressively to 12 months (for supplementary data, please visit our Web site at www.neurology.org and access the data through the Table of Contents page of the March 14 issue).

^{18}F-levodopa PET scan. The mean percent change in SOR of uptake of ^{18}F-levodopa by PET scan in 11 patients 12 months after surgery did not show a significant change on the side of the transplant (mean percent change ± SD: anterior putamen, unoperated side = 3.4 ± 19.4, surgical side = −1.6 ± 10.9; posterior putamen, unoperated side = −5.1 ± 10.5, surgical side = −6.2 ± 26.0; caudate, unoperated side = −2.4 ± 12.1, surgical side = 4.7 ± 11.0).

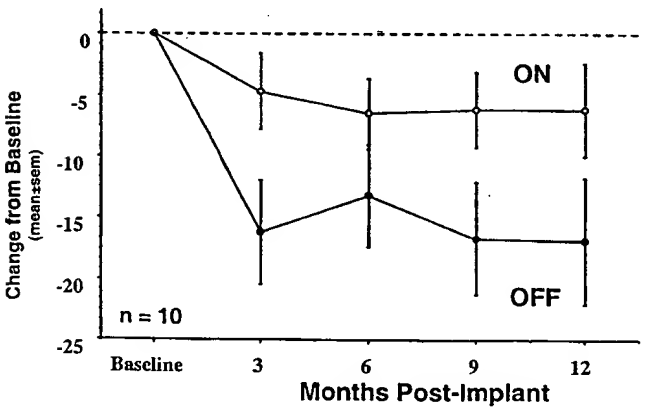

Figure. Total Unified Parkinson's Disease Rating Scale change from baseline (for 10 evaluable patients).

Discussion. In this study we report 1-year follow-up data of the first implantation of xenogeneic neural tissue into humans. Based on the reported therapeutic benefit of transplanting human embryonic VM tissue in patients with idiopathic PD, a pilot study was initiated to test the safety of implantation of embryonic porcine VM cells. Standardized PD clinical rating instruments were used to capture any changes in parkinsonian signs and symptoms. Unilateral implantation of a suspension of 12 million embryonic porcine VM cells into the striatum of patients with PD was well tolerated. There were no serious adverse effects directly related to the implantation of porcine embryonic cells, and no evidence of transmission of porcine-derived pathogens or a porcine-specific endogenous retrovirus. There was

evidence, in some patients, of improvement in standardized PD rating instruments.

The clinical results of approximately 40 PD patients who have received human embryonic allograft transplantation have been reported in the peer-reviewed literature. Different surgical approaches have been employed: unilateral or bilateral placements of tissue; placement of tissue in caudate, putamen, or both; preparation of tissue as cell suspensions or solid pieces; and amount of tissue placed. Despite these variations in surgical delivery techniques, some general conclusions can be made. Clinical improvement has been variable, ranging from mild to substantial, and it has been possible to withdraw L-DOPA medication in the most successful cases.[6] After unilateral transplantation, improvement in motor function is often bilateral, and clinical improvement is most consistently observed in the "off"-period state.[4-9] Improvement in symptoms of idiopathic PD or MPTP neurotoxin-induced parkinsonism is usually observed by 3 to 6 months, although in one case, substantial clinical improvement was not observed for 12 months.[4-9]

The results of the porcine unilateral VM transplantation reported here are similar to the first experience in unilateral human embryonic allograft transplantation, which used small amounts of neural tissue.[25-27] In the current study, the changes in standardized PD clinical rating instruments after unilateral transplantation of 12 million embryonic porcine VM cells were variable. Three patients have experienced substantial improvement in clinical rating scores (Patients 3, 7, and 11; see table 3); in others, improvement has been less marked, or there has been no clinically meaningful improvement. There was evidence of bilateral improvement in motor function, as measured by improvement in timed motor test scores. Improvement was most consistently observed in "off"-period UPDRS scores (see table 2). Clinical improvement was observed by 3 months. In Patient 1, improvement in a timed walking test was not observed until 6 months, and in Patient 7 apparent incremental effects on UPDRS "off" (for supplementary data, please visit our Web site at www.neurology.org and access the data through the Table of Contents page of the March 14 issue) were observed between postoperative month 3 (score = 55.0 seconds) and month 12 (score = 41.5 seconds). A delayed clinical response was observed in a patient who received a unilateral human embryonic transplant.[6] Whereas the explanation for this delayed effect in the current trial is not known, the progressive change in the human fetal transplant patient suggests that graft maturation may be required for some patients to achieve the maximal clinical response.

The change in PD rating scores observed in this group of porcine xenograft recipients does not appear to be due to a change in antiparkinson medications. There was no change in the mean DOPA equivalents between baseline and 12 months. Two of three patients who showed the greatest clinical improvement increased their antiparkinson medications slightly (for supplementary data, please visit our Web site at www.neurology.org and access the data through the Table of Contents page of the March 14 issue). A medication-related effect is unlikely to account entirely for the improvement in PD clinical rating measurements in these patients. Improvement in walking speed for Patient 1 was evident at a time when antiparkinson medication was not increased (for supplementary data, please visit our Web site at www.neurology.org and access the data through the Table of Contents page of the March 14 issue), and clinical improvement in Patient 7 was substantial at 9 months, when antiparkinson medications were increased only 10% (for supplementary data, please visit our Web site at www.neurology.org and access the data through the Table of Contents page of the March 14 issue). Patient 11 exhibited the largest improvement in UPDRS scores, after a small reduction in antiparkinson medication (for supplementary data, please visit our Web site at www.neurology.org and access the data through the Table of Contents page of the March 14 issue).

Open, noncontrolled studies are susceptible to subject and observer bias. A recent double-blind, controlled trial of human fetal transplantation showed evidence of placebo effect, as some control patients[28] reported positive responses in a global rating score. In the study reported here, it is not possible to assess the contribution of observer and subject bias to the change in UPDRS scores. It seems unlikely, however, that the magnitude of change observed in the best responders (Patients 7 and 11) could be entirely attributable to observer/subject bias. A controlled, randomized, placebo-controlled trial or porcine xenografts in PD patients will be required to address this issue.

Cyclosporine has been reported to enhance dopaminergic function[29] and, therefore, could theoretically contribute to the clinical benefit that has been observed in some patients who received porcine embryonic VM tissue and concurrent cyclosporine immunosuppression. The clinical improvements cannot be attributed solely to prodopaminergic effects of cyclosporine, because patients in the anti-MHC I antibody group, which did not receive cyclosporine, demonstrated improvements in UPDRS score of a similar magnitude to that observed in the cyclosporine group (see table 3).

^{18}F-levodopa PET has been used as a measure of engraftment of human embryonic VM in PD patients.[9,26,27,30,31] In general, there is increased ^{18}F-levodopa uptake when there is successful engraftment, and the greater the increase in ^{18}F-levodopa uptake, the greater the clinical benefit and amount of tissue transplanted.[6,30,31] Remy et al. demonstrated a correlation between Ki values and clinical response, and suggested that a change in Ki beyond two standard deviations is necessary to obtain optimal clinical responses in human embryonic transplantation, but

there were insufficient data in this report to assess the sensitivity of ^{18}F-levodopa PET.[30] One year after implantation of porcine embryonic VM cells, there was no group difference in ^{18}F-levodopa uptake between the ipsilateral (transplanted) and contralateral caudate or putamen. In the grafted striata of the patients (Patients 1, 7, and 11) who demonstrated the largest clinical responses, ^{18}F-DOPA PET scans did not show an increase.

In the current study of porcine xenografts there was a lack of correlation between PET findings and clinical response. This result is surprising in view of the meaningful clinical improvement in Patients 1, 7, and 11. This discrepancy could be due to the lack of sensitivity of ^{18}F-levodopa PET to detect the number of dopaminergic cells that are sufficient for clinical improvement. In the current study, 4 million porcine VM cells were transplanted at each of three sites unilaterally in striatum; only two tracks were in the putamen, the motor portion of the striatum. In most studies of human embryonic grafts in PD, substantial increases in ^{18}F-levodopa PET signals have been observed only when more than two tracks of cell suspension or solid grafts were implanted in the putamen.[6,7,9,26,30] Moreover, recent human embryonic VM allograft surgical procedures, which have generally demonstrated consistent clinical benefit and positive correlations with ^{18}F-DOPA PET uptake, have implanted more embryonic tissue over more implantation sites, or bilaterally.[6,9] Taken together, the ^{18}F-levodopa PET results in this series of porcine xenograft patients are not consistent with survival of a large number of porcine dopaminergic neurons, but the number of surviving dopaminergic neurons may be sufficient for clinical improvement, at least in some patients. Patient 2 of this study, for whom the number of surviving dopaminergic neurons in the porcine graft is known, did not have an ^{18}F-DOPA PET scan before death. Until the relationships among clinical response, PET signal, and surviving DA neurons in transplantation are better understood, the explanation for the clinical–PET discrepancy will remain unknown.

The absence of increase in ^{18}F-DOPA PET scans could also be due to poor survival of dopaminergic neurons as a consequence of immune-mediated mechanisms or lack of appropriate trophic factors. Patients in this study received a relatively low dose of cyclosporine, in order to maintain blood levels at approximately the level of an uncomplicated organ allotransplant. Greater immunosuppression (prednisone, azathioprine, and higher doses of cyclosporine), such as has been used in some human embryonic transplant programs,[6,7] may increase xenograft survival. Improvements in xenograft survival may be possible through additional modifications of the host immune response.[32] Neurotrophic and antiapoptotic enhancements of cell survival may also be beneficial in increasing embryonic cell survival.[33]

In this study, clinical improvement was observed in patients who underwent embryonic cell implantation with cyclosporine immunosuppression or who received cells that were treated, before implantation, with a monoclonal antibody directed at the MHC class I antigens (see table 3). This immunomodulation technique is believed to interfere with the process by which the T cells recognize and destroy engrafted cells[20] and may prevent T-cell recognition or modify T-cell responses,[34] such that rejection of a porcine embryonic VM xenograft is antagonized.[19] To our knowledge, this is the first example of use of this immunomodulation technique in humans. The improvement in performance on the standardized PD rating instruments observed in Patient 11 (see table 3) suggests that this immunomodulation technique may have utility in transplantation in humans, providing "local" immunosuppression and obviating the need for generalized immunosuppression. Based on these results, further clinical evaluation of this method of immunosuppression appears warranted.

The second patient in this study (cyclosporine group) died 7 months postsurgery due to pulmonary emboli. Histopathologic study of the brain demonstrated viable porcine dopaminergic neurons in the putamen at three injection sites.[23] The neurons appeared to be integrated within the host neuropil. There was no evidence of active rejection and little or no signs of inflammation at the transplant sites. These findings not only demonstrate that xenotransplantation of neural tissue into humans is feasible but also may provide a basis for the therapeutic effect observed in this study.

Pigs have been used as a source of transplantable tissue for heart valves, pancreatic islet cell transplants, skin grafts, and extracorporeal kidney and liver perfusion, but there have been relatively few cases of implantation of living xenogeneic tissue into humans. Until very recently,[35] follow-up safety and efficacy results have been reported in only selected patients.[36,37] With transplantation of tissue across species there is a risk of transmission of infectious pathogens from the animal source (zoonoses) or of microorganisms that are pathogens only by virtue of their association with the xenogeneic tissue (xenozoonoses).[15] While this study was in progress, a pig retrovirus, which is capable of replication in some human cells lines in vitro, was identified.[16] Although other C type retroviruses have been associated with lymphoproliferative and neurologic diseases, there is no evidence that porcine endogenous retrovirus is associated with disease in pigs or other species. Nonetheless, the possibility of transmission of a PERV from porcine tissue to human recipient warranted development of a sensitive assay to detect PERV DNA and monitoring of PBMCs from xenograft recipients for presence of PERV DNA. There was no evidence of PERV DNA in patient blood samples taken from 6 to 24 months after porcine embryonic VM implantation. Similar negative results for PERV transmission to humans have recently been observed in 10 patients who received porcine pancreatic islets,[36] in two renal dialysis patients whose cir-

culation had been linked extracorporeally to pig kidneys,[37] in 12 patients with Huntington's disease who received intrastriatal implantation of embryonic porcine neural tissue,[38] and in a large number of patients with other exposure to living porcine tissue.[35] Based on current results, it is reasonable to conclude that the PERVs will not show the high levels of transmission characteristic of some viruses. Longitudinal monitoring of patients who have been exposed to porcine tissue will add valuable information regarding the possible transmissibility and pathogenicity of PERV in humans. Taken together with the lack of serious adverse events related to the porcine cells and implantation procedure in the small number of patients in this pilot study, porcine cell implantation appears to have a favorable short-term safety profile.

These results demonstrate that unilateral implantation of embryonic porcine VM tissue into patients with advanced PD is well tolerated at 1 year. There was no evidence of transmission of porcine pathogens or PERV. Improvement in standardized PD clinical rating instruments was variable and similar to the results observed after the first unilateral human embryonic cell transplantation procedures, which used smaller amounts of embryonic tissue than are currently used.[6,9,21,25,30] Apparent clinical benefit was observed in three patients who received either cyclosporine immunosuppression or anti-MHC I monoclonal antibody–treated porcine VM cells, suggesting that both methods of immunosuppression may be useful in permitting porcine xenograft survival in humans. Controlled, randomized clinical trials in which a larger number of porcine VM cells are implanted bilaterally will be necessary to assess clinical benefit and safety more fully.

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Prospective validation of Consensus criteria for the diagnosis of dementia with Lewy bodies

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Article abstract—*Objective:* To determine the validity of a clinical diagnosis of probable or possible dementia with Lewy bodies (DLB) made using International Consensus criteria. *Background:* Validation studies based on retrospective chart reviews of autopsy-confirmed cases have suggested that diagnostic specificity for DLB is acceptable but case detection rates as low as 0.22 have been suggested. *Methods:* We evaluated the first 50 cases reaching neuropathologic autopsy in a cohort to which Consensus clinical diagnostic criteria for DLB, National Institute for Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association criteria for AD, and National Institute of Neurological Disorders and Stroke–Association Internationale pour la Recherche et l'Enseignement en Neurosciences criteria for vascular dementia (VaD) had been prospectively applied. *Results:* Twenty-six clinical diagnoses of DLB, 19 of AD, and 5 of VaD were made. At autopsy, 29 DLB cases, 15 AD, 5 VaD, and 1 progressive supranuclear palsy were identified. The sensitivity and specificity of a clinical diagnosis of probable DLB in this sample were 0.83 and 0.95. Of the five cases receiving a false-negative diagnosis of DLB, significant fluctuation was present in four but visual hallucinations and spontaneous motor features of parkinsonism were generally absent. Thirty-one percent of the DLB cases had additional vascular pathology and in two cases this contributed to a misdiagnosis of VaD. No correlations were found between the distribution of Lewy bodies and clinical features. *Conclusion:* The Consensus criteria for DLB performed as well in this prospective study as those for AD and VaD, with a diagnostic sensitivity substantially higher than that reported by previous retrospective studies. DLB occurs in the absence of extrapyramidal features and in the presence of comorbid cerebrovascular disease. Fluctuation is an important diagnostic indicator, reliable measures of which need to be developed further. **Key words:** Dementia—Lewy bodies—Clinical criteria—Sensitivity and specificity.

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Early, accurate clinical diagnosis of dementia with Lewy bodies (DLB) is important[1-7] because it is common, accounting for 15 to 36% of demented cases at autopsy,[8-10] and has a different course,[11] prognosis,[12] and treatment response[13,14] compared with other dementia types. Particularly important management issues include the avoidance of severe neuroleptic sensitivity reactions, achieving the optimal level of antiparkinsonian treatment without exacerbating psychiatric symptoms, and a possible beneficial response to cholinesterase inhibitors.

Three retrospective chart reviews examining the validity of the Consensus criteria for clinical diagnosis of DLB[7] have been published.[5,6,10] Reported sensitivity rates for a clinical diagnosis of probable DLB have varied from 0.22 to 0.75, and specificity from 0.79 to 1.0. Although such retrospective validation studies give a preliminary indication of the perfor-

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EXHIBIT F

Renal Heterotransplantation in Man *

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THE CONCEPT of cross-species transplantation has intrigued the mind of man for as long as he has recorded his myths and his history. Daedelus, who grafted bird feathers to his arms, was perhaps the first to transplant across the species barrier successfully; a similar experiment by his son, Icarus, ended in acute graft rejection when he flew too close to the sun.[1]

Scientific reports on renal heterotransplantation into man appeared early in this century. In 1905 Princeteau [2] inserted slices of rabbit kidney into a nephrotomy on a child with renal insufficiency. "The immediate results were excellent," he wrote. "The volume of urine increased; vomiting stopped. . . . On the 16th day the child died of pulmonary congestion . . ." (Fig. 1). In the following year, 1906, Jaboulay [3] on two occasions attempted renal heterotransplantation into man using vascular anastomoses. The heterografts, one from a pig and another from a goat, were inserted into the antecubital spaces. Neither graft functioned, and failure was attributed to vascular thromboses (Fig. 2).

In 1910 Unger [4] described his attempt at transplantation of kidneys from a non-human primate into man. The patient died 32 hours after transplantation, and autopsy showed venous thromboses (Fig. 3). Neuhof,[5] in 1923, attempted treatment of a patient with mercury bichloride poisoning by renal heterotransplantation. When he was unable to obtain a human kidney, he transplanted the kidney of a lamb into the patient. The patient died nine days later, but Neuhof was not totally discouraged. He wrote "(this case) proves, however, that a heterografted kidney in a human being does not necessarily become gangrenous and the procedure is, therefore, not necessarily a dangerous one, as had been supposed. It also demonstrates that thrombosis or hemorrhage at the anastomosis is not inevitable. I believe that this case report should turn attention anew . . ." (Fig. 4).

However, scientific interest in transplantation declined when the immunologic basis of the rejection process was established. With the demonstration of effectiveness of immunosuppressive drugs,[6-11] there has been renewed interest in transplantation. An accelerated effort in renal homotransplantation has been accompanied by problems in procuring organs. Ethical consider-

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Y. Greffe rénale.

M. PRINCETEAU présente les deux reins d'un enfant de son service et fait part à la Société de l'observation suivante :

Un enfant atteint d'ostéomyélite de l'extrémité supérieure du fémur droit ayant fusé dans l'os iliaque entre à l'hôpital des Enfants dans un état très grave, ayant de l'albumine dans les urines et un anasarque très marqué. Immédiatement il fit une grande incision, nettoya le foyer principal et après quatre interventions successives l'état de l'enfant s'était amélioré. Subitement, en fin août, cet enfant fit de la paralysie faciale et un œdème généralisé, vomissant tout ce qu'il prenait et urinant à peine 20 grammes de liquide par jour.

Devant de si graves symptômes, M. Princeteau fit une néphrotomie à gauche et inclut dans le rein deux tranches de rein d'un lapin. Les résultats immédiats furent excellents, l'urine augmenta, les vomissements cessèrent; quinze jours après l'intervention la quantité d'urine était de un litre par jour. Le seizième jour, l'enfant succombait à une congestion pulmonaire.

A l'autopsie, on trouva les deux reins gros et blancs, un foie musqué et la rate extrêmement ramollie. Quant aux inclusions de tranches de rein de lapin, elles paraissaient greffées, mais un examen histologique est nécessaire pour montrer la nature de l'adhérence. J. M.

FIG. 1. Princeteau, M.: Greffe rénale. J. Méd. Bordeaux, 26:549, 1905.

BULLETIN.

BULLETIN DU LYON MEDICAL

GREFFE DE REINS AU PLI DU COUDE PAR SOUDURES ARTÉRIELLES ET VEINEUSES.

J'ai pratiqué la greffe rénale par sutures vasculaires, deux fois, le 24 janvier et le 9 avril de cette année; je me proposais d'établir une suppléance fonctionnelle pour la sécrétion urinaire, en installant un rein étranger mais sain, pouvant venir en aide aux organes naturels qui étaient atteints de maladies incurables.

La première fois c'était chez une femme de 48 ans, brightique, présentant une forte hypertension, avec céphalalgie, diminution de la vue et de l'ouïe, et qui n'émettait par jour que 500 cc. environ d'une urine albumineuse et ne contenant que 4 gr. d'urée. Un rein de porc qui avait été tué trois heures avant, mis immédiatement après son extraction dans du sérum artificiel tiédi, fut anastomosé par ses vaisseaux aux vaisseaux du pli du coude de la malade. C'était le rein gauche et le pli du coude gauche. Une incision longitudinale, faite suivant la direction de l'artère humérale à ce niveau, mit à nu d'abord la veine médiane céphalique qui fut disséquée, puis profondément l'artère avant sa bifurcation. Une bande d'Esmarch avait été placée à la racine du bras, une ligature ayant été mise sur la portion des vaisseaux qui devait être leur bout périphérique, ceux-ci furent sectionnés, montrant leur bout central vide de sang. Alors le rein fut fixé dans cette plaie, la face antérieure en avant et non recouverte, l'uretère occupant le plan profond et dirigé vers le bord interne du pli du coude. L'artère rénale fut soudée au bout central de l'artère humérale, la veine rénale au bout central de la veine médiane céphalique, de la façon suivante : chacun des vaisseaux de la malade était introduit dans la lumière d'une virole métallique, de dimensions appropriées, puis retournée à l'extrémité de cette virole jusqu'à une rigole circulaire où il était fixé par un fil circulaire; la paroi interne du vaisseau, endartère ou endoveine, devenait ainsi externe et pouvait être comptée avec la

FIG. 2. Jaboulay, M.: Greffe de reins au pli de coude par soudures artérielles et veineuses. Lyon Med., 107:575, 1906.

Aus der experimentell-biologischen Abteilung des Königl. pathologischen Instituts der Universität Berlin und aus der Privatklinik Dr. Ernst Unger.

Nierentransplantationen.

(II. Mitteilung.)

Von

Dr. Ernst Unger-Berlin.

(Nach einem am 2. März 1910 in der Berliner medizinischen Gesellschaft gehaltenen Vortrage.)

M. H.! Im April 1909 hatte ich mir erlaubt, Ihnen über Nierentransplantationen zu berichten. Carrel und Guthrie ge-

FIG. 3. Unger, E.: Nierentransplantationen. Klin. Wschr., 47:573, 1910.

THE TRANSPLANTATION OF TISSUES

BY

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WITH THE COLLABORATION OF

SAMUEL HIRSHFELD, M.D.

SURGICAL MONOGRAPHS

UNDER THE EDITORIAL SUPERVISION OF

DEAN LEWIS, A.B., M.D. EUGENE H. POOL, A.B., M.D.

ARTHUR W. ELTING, A.B., M.D.

D. APPLETON AND COMPANY
NEW YORK LONDON
1923

FIG. 4. Neuhof, H.: Transplantation of Tissues. New York: Appleton and Co., 1923, p. 260.

TABLE 1

Name	Donor ABO	Donor Rh	Donor MN	Recipient Patient	Recipient ABO
Adam	$A_{1,2}$	ccD-	M	1. J. D.	A_1
Dave	O	ccD-	MN	2. P. R.	O
Butch	O	ccD-	*	3. E. P.	B
Topeka	$A_{1,2}$	ccD-	M	4. D. P.	O
Fred	O	ccD-	MN	4. D. P.	O
Vickie	A_2	ccD-	M	5. A. A.	A_2
James	A_2	ccD-	*	6. O. S.	A_1

* Not done.

ations have posed difficult problems, particularly in the use of volunteer human donors. The use of organs harvested from human cadavers has depended on rapid transfer or preservation and has imposed restrictions of supply, selection and scheduling.

In our renal homografting program we had increasing difficulty obtaining donor organs. Attempts to use cadaveric kidneys met with no prolonged success, and the supply of expended kidneys was inadequate. We were reluctant to press the use of volunteer humans for ethical, scientific and legal reasons.

As this impasse was developing we decided to explore the use of non-human sources for clinical renal transplantation. This decision was prompted, in part, by clinical urgency. Additionally, a regional primate center in this vicinity brought scientists experienced in primatology. Furthermore, an active program in transplantation immunology had been developed to give an added base to the study.

The fact that no significant, sustained success had accompanied any previous attempt[2-6, 12, 13] at renal heterotransplantation into man was disquieting but hardly conclusive that failure was inevitable. Much recent evidence had suggested that graft-rejection is not always a simple, all-or-none process, but rather is a spectrum of phenomena, varying from organ to organ and species to species. Furthermore, the pessimism which has beclouded heterotransplantation had been derived largely from skin-grafting studies between widely disparate species. It seemed unwarranted to project these findings into the field of renal transplantation among primates, one of whom is man.

Our basic conjecture was that kidneys from non-human sources closely related to man would respond similarly to human kidneys following transplantation into man. The problem of more strenuous immune suppression was balanced against the advantages in the use of a non-human donor.

In practice, all patients were terminal uremics, maintained on dialysis, who were presented the following alternatives:

1) Supporting treatment only, 2) Homograft from a human volunteer, with the word "volunteer" defined in the strictest sense, 3) Cadaveric homograft, if and when available, or 4) Heterograft.

The risks, the uncertainties, the experimental nature of the work were discussed with the patients and their families. If they chose to procede with transplantation and had no volunteer donor, a search was made for a cadaveric kidney. If no suitable cadaver kidney became available in a stated period of time, a heterograft was used, with the patient's understanding and consent.

The chimpanzee was selected as the donor in heterografting studies for several reasons. This species is considered taxonomically closely related to man. The range of size of chimpanzees approximates that of man, a factor which might have significance in the transplantation of other organs in addition to kidneys. Furthermore, renal function of chimpanzees corresponds closely to that of man.[14] Additionally, chimpanzees have been found to be of blood types A and O,[15] thereby offering the possibility of the *universal donor* from the standpoint of blood groups.

Case Reports

Between November 5, 1963 and February 10, 1964 six patients received renal heterotransplants from chimpanzees. All patients were in terminal uremia necessitating dialysis and all patients received pre-transplantation treatment of azathioprine, actinomycin C and steroids. Selection of the donor was based on body size and blood typing of donor and recipient (Table 1). In each instance the donor received general endotracheal anesthesia, with monitoring of blood pressure, electrocardiogram and body temperature. Creatinine clearance was determined in each donor. At moderate hypothermia (about 30° C.) the entire renal complex, including both kidneys and ureters, aorta, and vena cava, was removed *en bloc* after anticoagulation and was irrigated. Patients were prepared simultaneously by extraperitoneal exposure of the external iliac artery and vein. In each instance the aorta and vena cava of the graft were anastomosed to the recipient's external iliac artery and vein, respectively, in an end-to-side fashion (Fig. 5). The periods of ischemia, from the time of vessel clamping in the donor until blood flow was restored through the graft in the recipient, varied from 36 to 43 minutes. All patients received postoperative azathioprine, actinomycin C, steroids and x-radiation to the transplant.

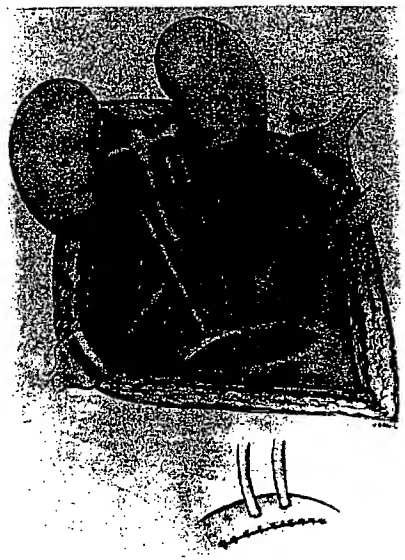

FIG. 5. Artist drawing of the operative field. The renal complex from the donor is implanted into the extra-peritoneal space in the recipient. The ends of the donor aorta and the vena cava are anastomosed to the sides of the external iliac artery and vein, respectively. The ureters are implanted into the bladder through sub-mucosal tunnels.

Case 1. This 43-year-old former dock worker with a history of hypertension since 1957, was admitted to the Veterans Administration Hospital, New Orleans, in 1959. Renal biopsies showed nephrosclerosis and chronic glomerulonephritis. He was treated with dietary management, including salt restriction. He was readmitted in June, 1963 because of progressive uremia, hypertension, and congestive heart failure. Laboratory studies included the following: BUN, 240 mg.%, creatinine, 14 mg.%, and creatinine clearance 8 ml./min. There was no improvement with dietary management, and peritoneal dialysis was required.

On November 5, 1963 he received a renal heterograft. During the first 14 hours after transplantation the urinary output was 6,700 cc. The BUN which was 112 mg.% on the day of operation, decreased to 39 mg.% by the fourth day following transplantation. The creatinine which was 11.2 mg.% on the day of operation fell to 1.5 mg.% 48 hours after transplantation. Four days after transplantation threatened rejection occurred but was reversed following local irradiation to the graft and increased doses of immunosuppressive drugs (Fig. 6). His early course has been reported previously in detail.[16] Function of the graft was confirmed by renograms, scans and intravenous urogram (Fig. 7).

On December 18th he was allowed to leave the hospital because he was asymptomatic and had normal renal function. He was readmitted on December 20th with a temperature of 39.4° C. and radiographic evidence of an infiltrate in the right middle lobe with pleural effusion. Culture of the sputum revealed *Aerobacter aerogenes*. The dosage of azathioprine was lowered because of leukopenia, but renal function continued satisfac-

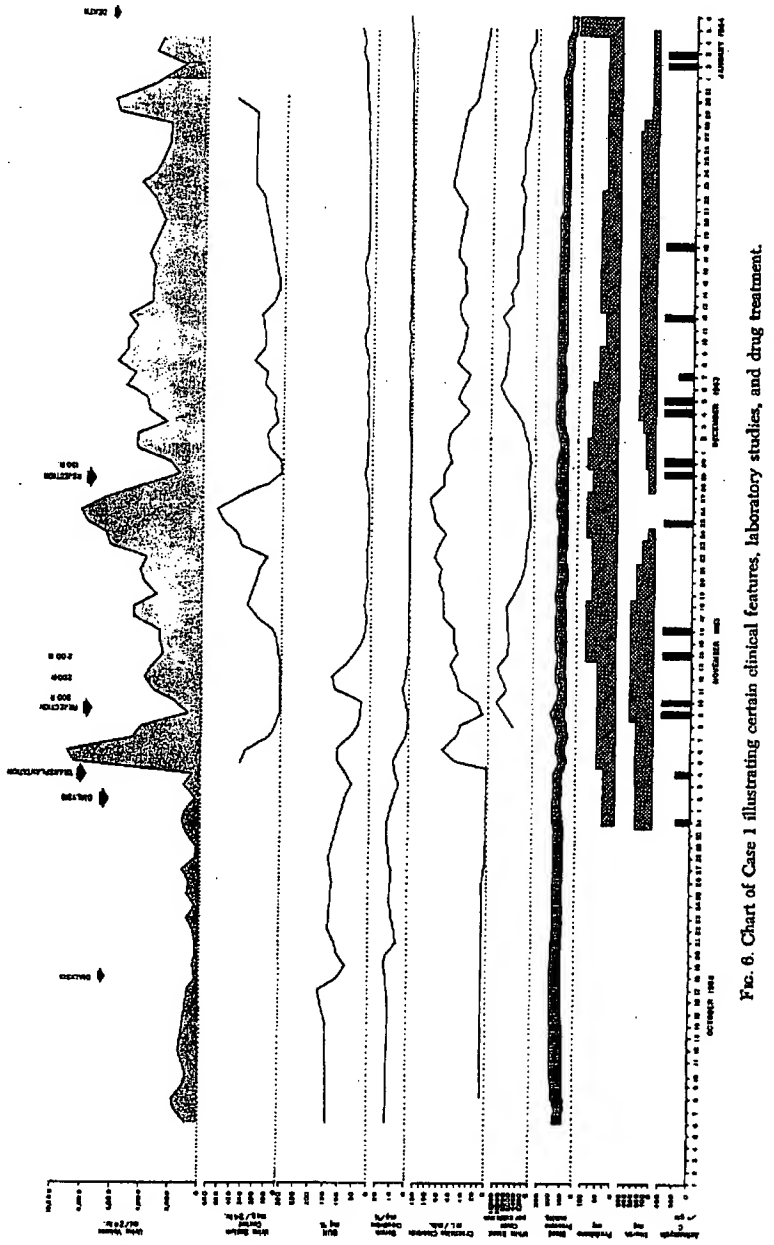

FIG. 6. Chart of Case 1 illustrating certain clinical features, laboratory studies, and drug treatment.

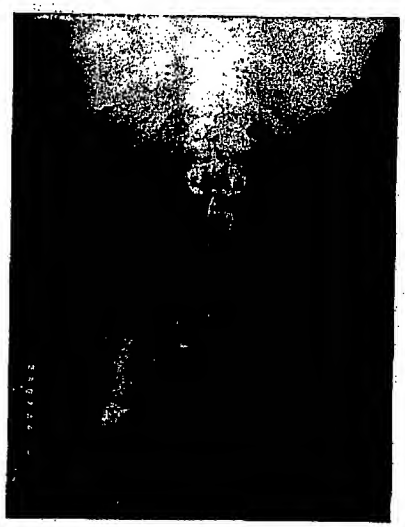

FIG. 7. Intravenous urogram in Case 1 performed ten days following transplantation. Film demonstrates contrast material in calyces of both transplanted kidneys and in both ureters.

tory. The patient's condition later deteriorated rapidly and he died 63 days after transplantation, following a period of shock apparently due to sepsis.

FIG. 8. Photograph of transplanted kidneys in Case 1. Arterial and venous anastomoses were patent.

Autopsy showed acute bronchopneumonia, right lower lobe; acute tracheobronchitis; resolving abscess, right middle lobe. The transplanted kidneys showed acute tubular necrosis, consistent with shock; there was no cellular infiltrate or changes in the blood vessels (Fig. 8, 9).

Serial renograms (Fig. 10) demonstrated a progressive delay in the appearance of the peak of uptake. Changes in the renogram, however, were not correlated with biochemical changes in renal function.

Hemagglutination studies (Fig. 11) demonstrated a precipitous rise in titer beginning on the fourth day following transplantation. This titer fell to pre-transplantation levels at the end of one month and remained at this level throughout the

FIG. 9. Photomicrograph of transplanted kidney from Case 1. Sections show interstitial edema, tubular degeneration, normal glomerulus and intra-lobular artery.

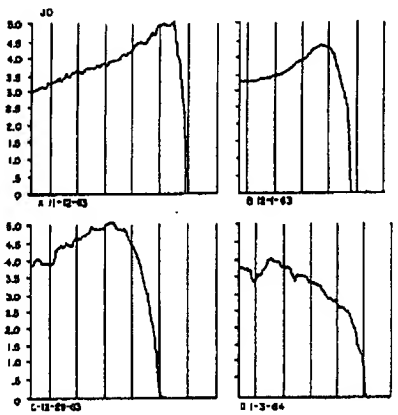

FIG. 10. Serial renograms in Case 1.

second month. Data on cytotoxicity studies are shown (Table 2).

Case 2. This 12-year-old patient was admitted to Charity Hospital on September 9, 1963 with a history of enuresis and repeated bouts of urinary tract infection due to bladder neck obstruction. Previously bladder neck resections and left nephrostomy had been performed. Prior to admission he had several episodes of convulsions and coma, one of which left him with right-sided hemiparesis.

Physical examination revealed a chronically ill patient with a B.P. of 170/116. The abdomen showed scars of previous operations and a left nephrostomy. The clinical impression was chronic pyelonephritis, secondary to bladder neck obstruction, with terminal uremia.

Studies included serum creatinine of 15.4 mg.%, BUN 172 mg.%, hematocrit 19%. On 11-8-63, after preparation with peritoneal dialysis, bilateral nephrectomy and splenectomy were performed. Thereafter the patient was maintained on weekly hemodialysis, using a shunt in the right forearm. On 12-23-63, heterotransplantation was performed. The bladder was contracted, with a thickened wall. On 12-24-64 his urinary output dropped via the urethral catheter, and increased drainage of urine was noted from the lower end of the wound. On 12-25-63 cutaneous ureterostomies were performed. In the early postoperative period urinary collections were inadequate for technical reasons; however, urine volume remained 2,000–6,000 ml./day. Creatinine clearance immediately after operation was 60 cc./min. Three days after operation the BUN was 17 mg.% and serum creatinine was 0.7 mg.% (Fig. 12). On 12-28-63, spiking daily fevers of 104–105° F. developed.

TABLE 2. *Cytotoxicity Studies*

Case 1						
Days after transplantation	3	10				
Cytotoxicity: % loss viability	0	10				
Case 3						
Days after transplantation	0	22	36			
Cytotoxicity: % loss viability	0	24	16			
Case 6						
Days after transplantation	0	10	17	26	27	28
Cytotoxicity: % loss viability	0	1	28	12	25	26

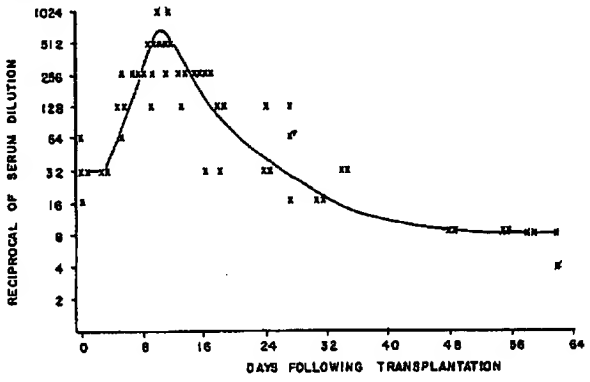

FIG. 11. Heterohemagglutination studies in Case 1.

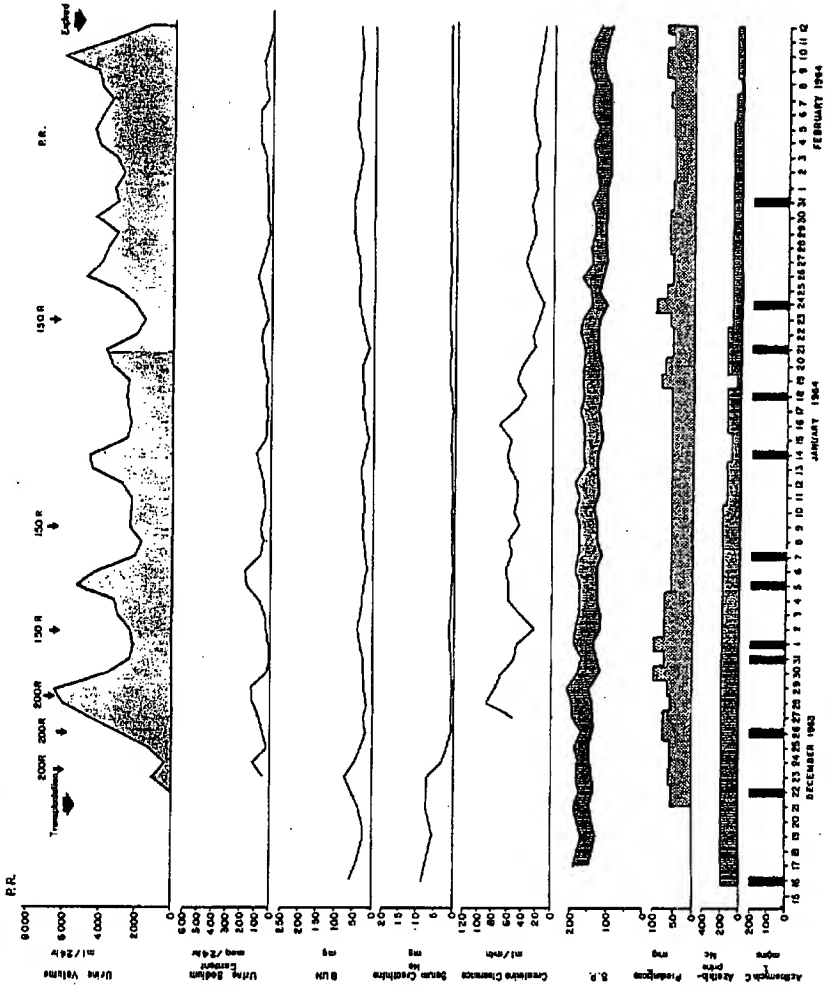

FIG. 12. Chart in Case 2 illustrating certain aspects of clinical course, laboratory studies, and drug treatment.

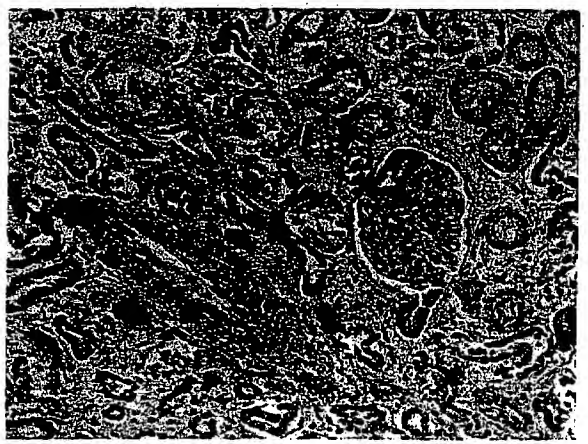

FIG. 13. Photomicrograph of transplanted kidneys from Case 2. Sections show interstitial edema, tubular degenerative changes, fibrin thrombi in the glomerular tufts and normal intralobular artery.

This was attributed to infection in the area of the transplant and in areas of the old operative incisions. During the last month of his life his white blood cell count and platelet count fell progressively; BUN remained in the range of 30–50 and serum creatinine 1.0–1.5 mg.%. His septic course, however, failed to respond to a variety of antibiotics. The predominant organism from the wound was *Aerobacter aerogenes*, not sensitive to antibiotics. He died on 2-13-64, approximately seven weeks after transplantation. Autopsy showed loculated pus around the transplant, at the site of nephrectomy, and in the abdominal incisions. Sections of the transplant showed acute tubular necrosis and edema without cellular infiltrate (Fig. 13).

Serial renograms (Fig. 14) demonstrated changes beginning in the second month following transplantation. These changes, however, were not closely correlated with the patient's clinical picture or chemical evidence of changes in renal function.

Agglutination studies demonstrated the absence of heteroagglutinans initially and throughout the entire post-transplantation course (Fig. 15).

Case 3. This 23-year-old school teacher was admitted in November, 1963 with chronic glomerulonephritis and progressive uremia. She had an episode of acute glomerulonephritis at 14 and persisting proteinuria. She had remained asymptomatic until approximately five months before admission when she noted weakness and dizziness.

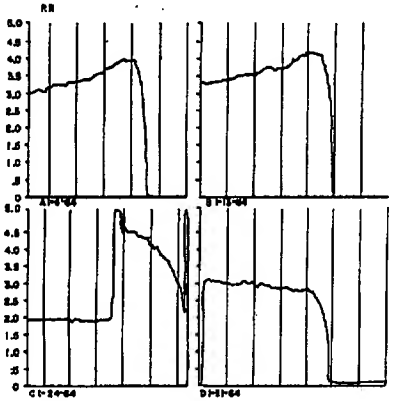

FIG. 14. Serial renograms in Case 2.

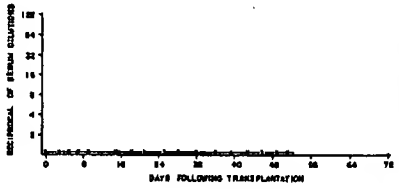

FIG. 15. Heterohemagglutinination studies in Case 2.

On admission her blood pressure was 190/120 and laboratory studies included BUN of 184 mg.%, creatinine of 40 mg.% and creatinine clearance of 4 ml./min. Rapid deterioration of her condition necessitated peritoneal dialysis.

On January 13, 1964 she received a renal heterotransplant. Diuresis occurred with a urinary output on the day of operation of 7,000 ml. By the third day following transplantation the BUN had fallen from a pre-transplant level of 116 mg.% to 12 mg.%, and the serum creatinine fell from a preoperative level of 21 mg.% to 0.9 mg.%. Creatinine clearance was 50 cc. per min. Her blood pressure fell to normotensive levels, 110/70. Her subsequent course demonstrated satisfactory renal function until the 23rd day following operation when threatened rejection was suspected (Fig. 16). Urinary output decreased to 1,000 ml./24 hours, and BUN and creatinine rose to 28 and 1.9 mg.%, respectively. Creatinine clearance fell to

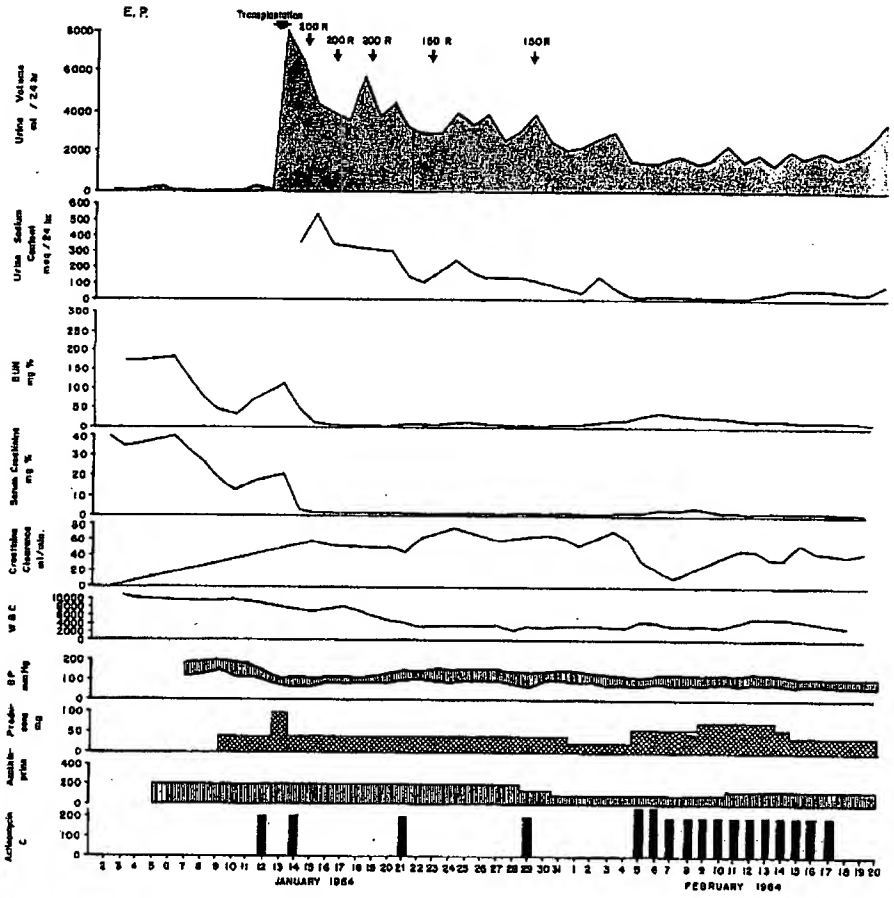

FIG. 16. Chart of Case 3 illustrating certain clinical features, laboratory studies, and drug treatment.

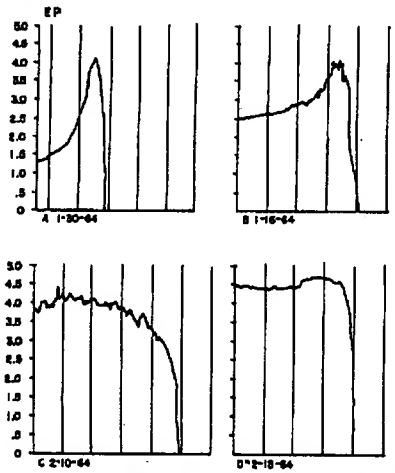

FIG. 17. Serial renograms in Case 3.

FIG. 18. Intravenous urogram in Case 3 performed 12 weeks after transplantation. In this case the transplant was inserted in such a way that the calyx of the upper kidney faces medially and the calyx of the lower kidney laterally. Contrast material is seen in both calyces and ureters.

31 ml. per min. and urinary sodium content to 11.6 mEq. for a 24-hour period. Gradual reversal of rejection occurred during the following two weeks, although unexplained fever persisted for 3 months. She is now asymptomatic and has normal renal function 6½ months after transplantation.

Serial renograms (Fig. 17) in this patient demonstrated a delay in peak activity which coincided with clinical and biochemical evidence of threatened rejection. Following reversal of rejection, the renogram resumed a more normal pattern. Intravenous urogram 12 weeks after transplantation showed function of both transplanted kidneys (Fig. 18).

Agglutination studies (Fig. 19) in this patient demonstrated a slight rise in titer at approximately three weeks after transplantation. The agglutination titer subsequently returned to previous levels.

Case 4. This 35-year-old man was referred to Charity Hospital in terminal uremia for renal transplantation. In 1955 a clinical diagnosis of chronic glomerulonephritis was confirmed by renal biopsy. Since that time he had gradually deteriorated. In August 1963, he became markedly uremic, his BUN rising to 250 mg.%.

Physical examination upon admission revealed a chronically ill man with B.P. 210/110. Examination of the thorax showed crepitant rales over both lung fields posteriorly. The heart was enlarged, a marked pericardial friction rub was heard over the entire pericardium, and a left ventricular gallop rhythm was audible. The liver was enlarged and tender. He showed bilateral costovertebral tenderness, edema of the ankles and sacral area, generalized muscular twitching and ataxia. The clinical impression was chronic renal disease with uremia, anemia and congestive heart failure. Studies on admission included an hematocrit of 18%, BUN 176 mg.% creatinine 16.8 mg.%. On 12-30-63 peritoneal dialysis was begun and

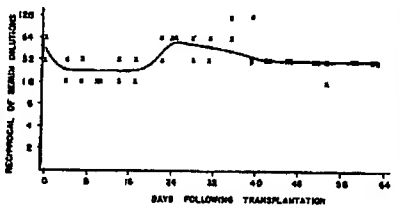

FIG. 19. Heterohemagglutinination studies in Case 3.

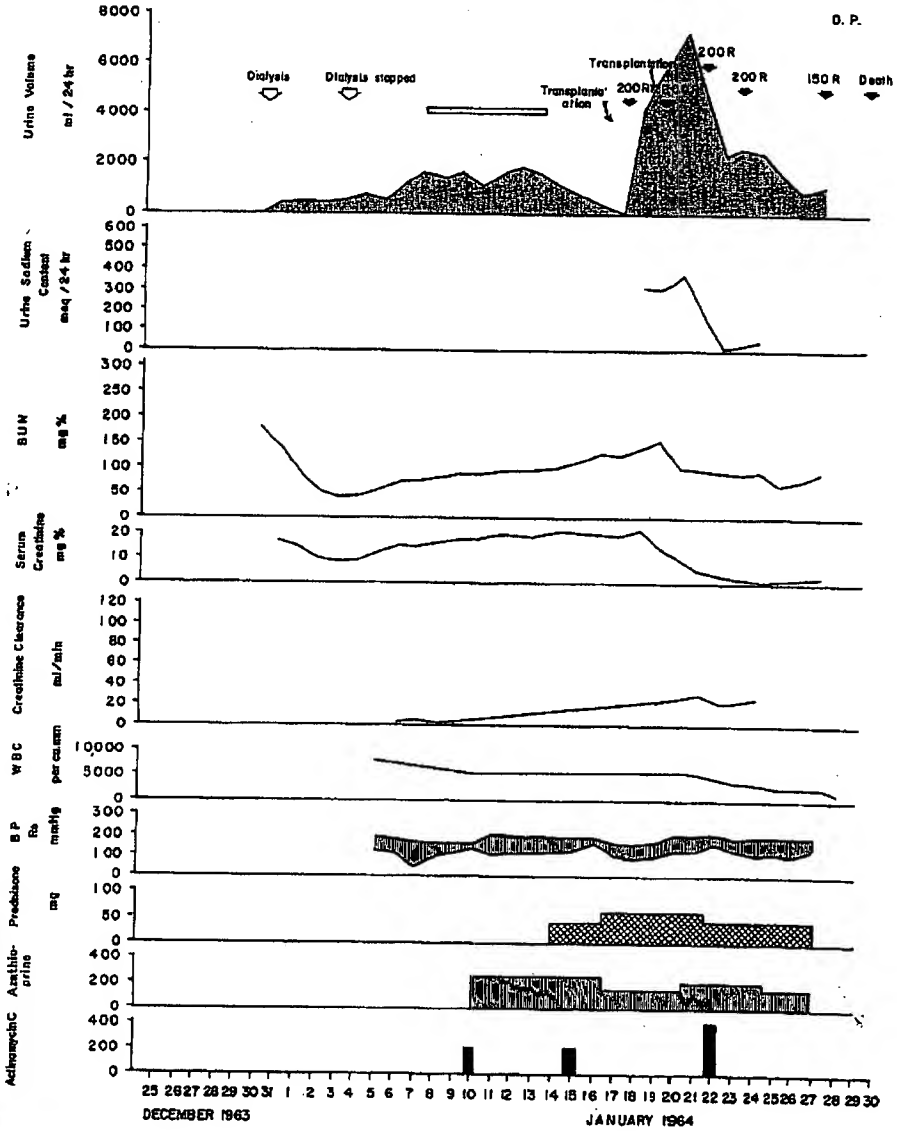

FIG. 20. Chart in Case 4 illustrating certain clinical features, laboratory studies and drug treatment.

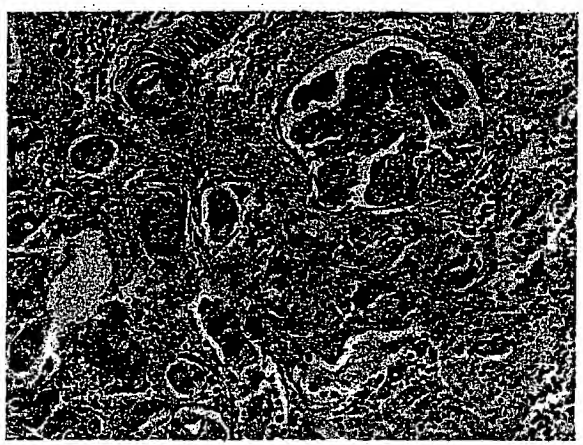

FIG. 21. Photomicrograph of the first transplant in Case 4, in which kidneys from a type A donor were implanted in a type O patient. Sections show severe tubular degeneration and interstitial edema, fibrin thrombi in a glomerulus, fibrinoid necrosis of blood vessels, and interstitial infiltration by lymphocytes and neutrophils.

continued for four days, with marked clinical and chemical improvement. On 1-16-64, the patient who was blood type O, received an heterotransplant from a chimpanzee of blood type A. No urine appeared from the ureters of the transplant and there was no significant output during the next 36 hours. On 1-18-64, a type O chimpanzee was obtained by special arrangement through the Air Force. The heterotransplant from the type O chimpanzee was placed in the opposite inguinal area and functioned immediately. The renal complex from the type A chimpanzee was removed. This wound was found to be grossly infected and was extensively drained. Examination of the removed specimen revealed it to be swollen and hemorrhagic. After operation, the patient ran an extremely septic course, and culture of his wound exudate and his blood grew out aerobacter which was resistent to all available antibiotics. His course after operation was marked by rapid deterioration to his death on 1-29-64, 11 days after the second transplantation. The creatinine clearance of the

FIG. 22. Photomicrograph of kidneys following second transplantation in Case 4. These kidneys were from a donor of blood type O. Sections show normal glomerulus with interstitial edema, tubular degenerative changes, and slight lymphocytic infiltration.

transplant after operation ranged from 20–30 ml./min. (Fig. 20). The serum creatinine during the early postoperative period fell rapidly to 2 mg.%. The BUN, however, which was 156 mg.% prior to operation never fell below 76 mg.%. There was a marked drop in his WBC to 1,000/cubic mm. and his platelet count dropped to 48,000. This was attributed to immunosuppressive drug therapy which was discontinued two days prior to death.

Pathologic studies of the transplant from the type A chimpanzee showed hypercellular glomeruli, tubular degeneration, edema, moderate cellular infiltration, and fibrinoid necrosis of blood vessels (Fig. 21). Sections of the graft from the type O donor showed normal glomeruli and vessels, with interstitial edema, tubular degenerative changes and slight cellular infiltration (Fig. 22). Hemagglutinin studies are illustrated (Fig. 23).

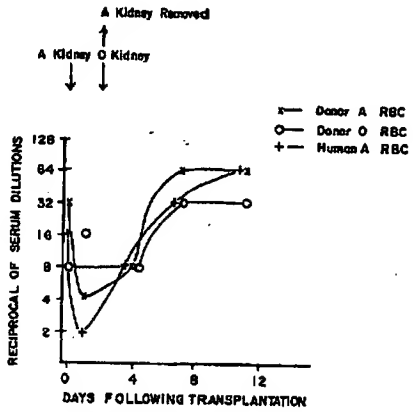

FIG. 23. Heterohemagglutinination studies in Case 4.

Case 5. This 48-year-old woman was admitted on 1-3-64 for evaluation for renal transplantation. Four years previously she was found to have polycystic renal disease, with hypertension, hematuria and back pain. During the six month period prior to admission she showed progressive renal failure and uremia.

Initial laboratory studies included blood urea nitrogen of 140 mg.%, serum creatinine, 14.8 mg.%, creatinine clearance, 5 ml./ min. Conservative treatment resulted in transient improvement, but subsequent deterioration and coma necessitated the initiation of peritoneal dialysis on 1-24-64.

On 2-4-64 she received a renal heterotransplant. Her early postoperative course was satisfactory with gradual improvement of renal function; creatinine clearance reached 80 ml./min. by the ninth postoperative day. However, on 2-14-64 she became febrile, and urine culture showed *Aerobacter aerogenes*. Marked leukopenia and thrombocytopenia developed (Fig. 24). On 2-22-64 threatened rejection was suspected and she was placed on higher doses of steroids and given additional local radiotherapy. Later that day she developed profound shock which failed to respond to the administration of steroids, antibiotics and vasopressors. Blood drawn just prior to her death grew *Aerobacter aerogenes*, and autopsy findings confirmed the impression of generalized sepsis.

Sections of the renal transplant showed normal glomeruli, marked tubular degeneration and edema, and perivascular infiltration with lymphocytes, and occasional plasma cells and histiocytes. An additional finding was fibrinoid necrosis of blood vessels (Fig. 25–28).

Agglutination studies showed an initial fall in titer followed by a rise on the fifth day and a subsequent decline. The alterations were not correlated with any obvious clinical or biochemical changes.

Case 6. This 16-year-old girl was admitted to Charity Hospital on 12-29-63 with a diagnosis of chronic glomerlonephritis and progressive uremia. For approximately one year she had noted dizziness, fatigability and intermittent periobital edema.

On admission laboratory studies included BUN 248 mg.%, creatinine 18.4 mg.% and creatinine clearance 4 ml./min. Peritoneal dialysis was begun on 12-30-63 and continued for one week.

On 2-10-64 she received a renal heterotransplant. The urinary output was 1,640 ml. and the creatinine clearance was 60 ml./min. on the first day following operation. Although the BUN and serum creatinine decreased to 22 mg.% and 0.6 mg.%, respectively, by the fourth day following operation, subsequently there was gradual increase of the BUN over the ensuing two-week period (Fig. 30). By 2-25-64 the BUN had reached a level of 112 mg.%, serum creatinine had increased to 4.1 mg.%, and creatinine clearance had diminished to less than 5 ml./min. despite vigorous immunosuppressive treatment.

A wound culture taken on 2-16-64 revealed *Aerobacter aerogenes*. On 2-29-64 the renal heterograft was considered nonfunctional and was removed. The wound was grossly infected and was drained. Despite peritoneal dialysis, and discontinuance of immunosuppressive drugs and treat-

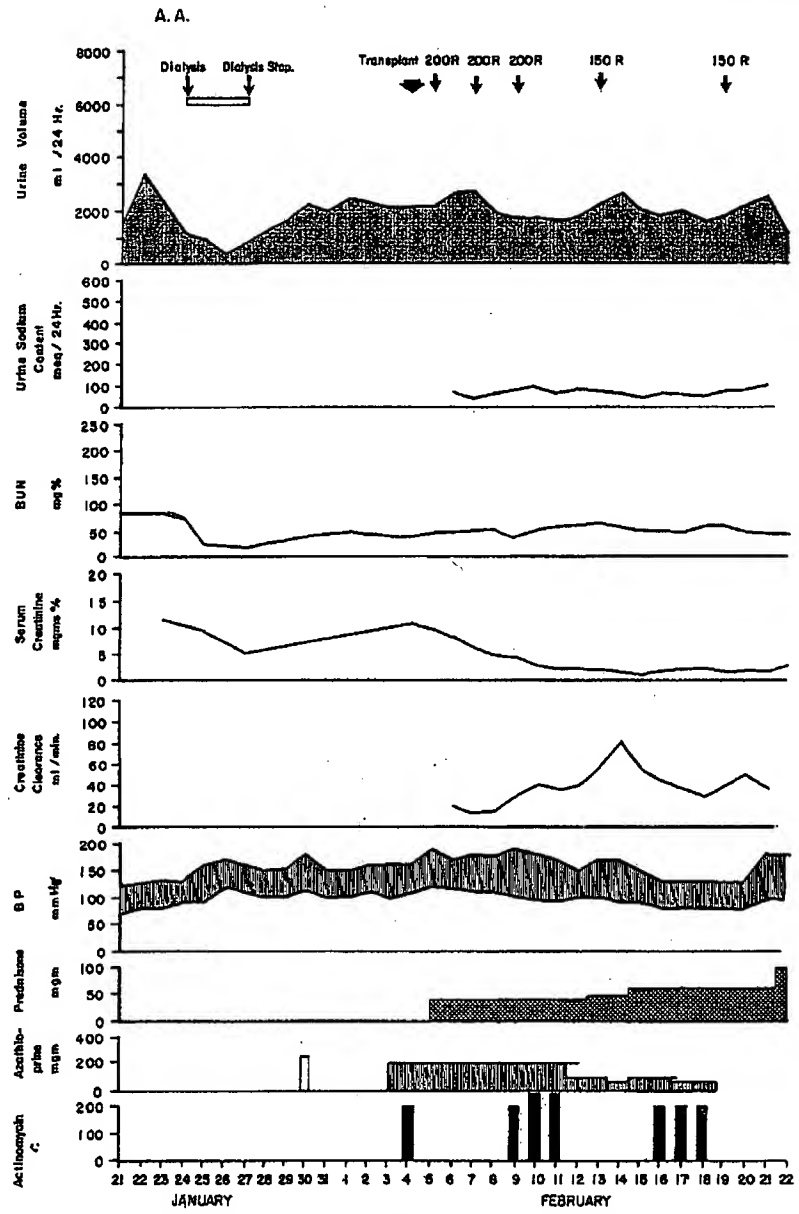

FIG. 24. Chart of case five illustrating certain clinical features, laboratory studies, and drug treatment.

FIG. 25. Photomicrographs of transplanted kidneys in Case 5. Sections show an intralobular artery with peri-vascular cellular cuffing. There is marked tubular degeneration and edema, without marked changes of the glomerulus.

ment with antibiotics she died 27 days following transplantation from uremia and convulsions.

Sections of the transplanted kidneys showed interstitial edema, tubular necrosis, glomerular hypercellularity and mild cellular infiltration (Fig. 31).

Agglutination studies demonstrated a rise in titer beginning approximately one week after transplantation (Fig. 32). This change coincided with clinical evidence of rejection. The subsequent decline in titer occurred approximately at the time the graft was removed. Pre-rejection levels of agglutination titers were observed when the patient was maintained on dialysis and immunosuppressive agents were discontinued.

Discussion

Limitations of scope and time of this study do not permit precise definition of many aspects of renal heterotransplantation in man. Nevertheless, several observations on this small group of patients studied for short periods of time deserve comment.

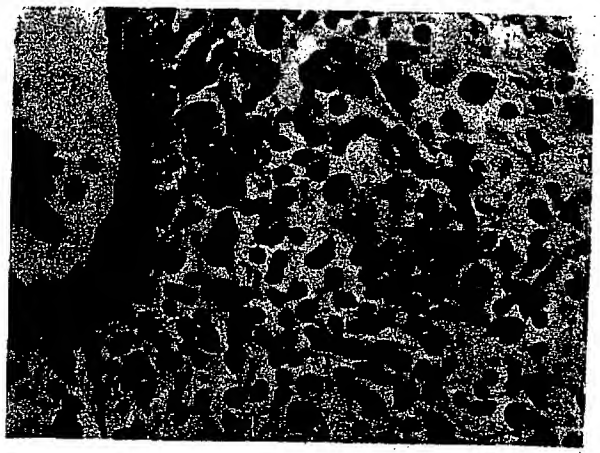

FIG. 26. High-power photomicrograph of perivascular cellular infiltrate showing lymphocytes with occasional plasma cells and histiocytes.

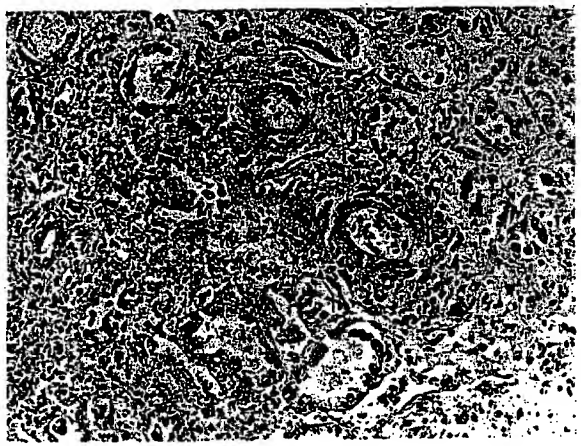

FIG. 27. Photomicrograph of transplanted kidneys in Case 5. Sections show fibrinoid necrosis of blood vessels, severe tubular degeneration and cellular infiltration.

Function of the transplant occurred in all cases with the exception of the instance in which major blood group incompatibility was challenged (Case 4, first transplant). The degrees of function varied considerably. Prompt diuresis and return toward normal of renal function often occurred, but in some instances this was delayed, perhaps due to tubular damage. In general, renal function has proved to be a lesser problem than infection attributed to immune suppression.

Initially an assumption was made that rejection of heterografts would be more immediate and severe than rejection of homografts. For this reason, strenuous immunosuppressive measures were used, including azathioprine, steroids, actinomycin

FIG. 28. High-power view of sections shown in Figure 27 from the transplanted kidneys in Case 5. There is severe fibrinoid necrosis of the blood vessel.

C and local irradiation. We have continued to use these modalities, but we have chosen to decrease our reliance on heavy doses of immunosuppressive drugs in view of our preponderant difficulties with infection compared with graft-rejection.

The interpretation of serial renograms has been difficult. A progressive delay in uptake of isotope has been observed, but such changes often were not associated with obvious alterations in renal function. In one instance (Case 3) a marked delay in uptake coincide with clinically apparent rejection. In this case the renogram returned toward a normal pattern after reversal of rejection.

Increasing effort has been placed on matching recipients and donors and on following the immunologic response in the recipient. All patients and donors have been typed for major blood groups, ABO, Rh and MN. Chimpanzees were found to show A_1, A_2, $A_{1,2}$ or O; uniformly ccD-Rh_o^{-ch}; and M or MN, erythrocytes with the corresponding complementary hemagglutinins occurring only in the ABO group. These results are in agreement with those of Wiener and Moor-Jankowski.[15] The designations A_1, A_2, or $A_{1,2}$ reflect differences in susceptibility to *Dolichos biflorus* extracts rather than identity with the human subgroups of A. All chimpanzee erythrocytes were agglutinated by rabbit anti-human M but showed partial or no agglutination in similar anti-N serum and are listed as MN or M on this basis. Furthermore, all chimpanzee red cells were strongly agglutinated in anti-C(hr') and anti-D (Rh_o) sera but failed to react with anti-C, anti-E or anti-e. Tests for other groups, such as Kk or Ss, which require the use of developing anti-human globulin anti-serum were not done because of the evident possibility of interference by human anti-chimpanzee heterohemagglutinins. These results can be interpreted to indicate strong similarities, but not identity, between human and chimpanzee Rh and MN isoantigens. Chimpanzee blood group A substance may be even more closely related to human A, in that one patient, (Case 4) responded to stimulus by a type A chimpanzee kidney with a specific increase in antihuman A_2.

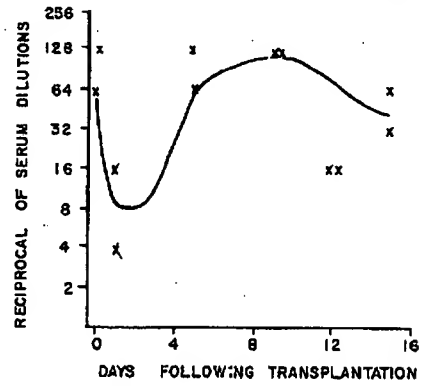

FIG. 29. Heterohemagglutination studies in Case 5.

Cytotoxicity tests, performed by the trypan blue dye exclusion method were used to determine viability. Chimpanzee (type O) lymph node cells obtained at the time of transplantation were exposed to serum, washed, and then exposed to complement to eliminate any anti-compremental effect of the serum. Earlier tests of the same samples were negative when this precaution was not taken. All sera reported were tested at the same time against one donor. No normal serum or pretransplant serum showed any activity, and viability in all control tubes was 90 per cent or greater throughout. Hemagglutinations were performed by standard technics using either chimpanzee donor erythrocytes or a battery of chimpanzee red cells of the same ABO type.

The patient in Case 1 showed an abrupt rise in anti-A^{ch} rbc heterohemagglutinins at days 5–10 following transplantation, rising

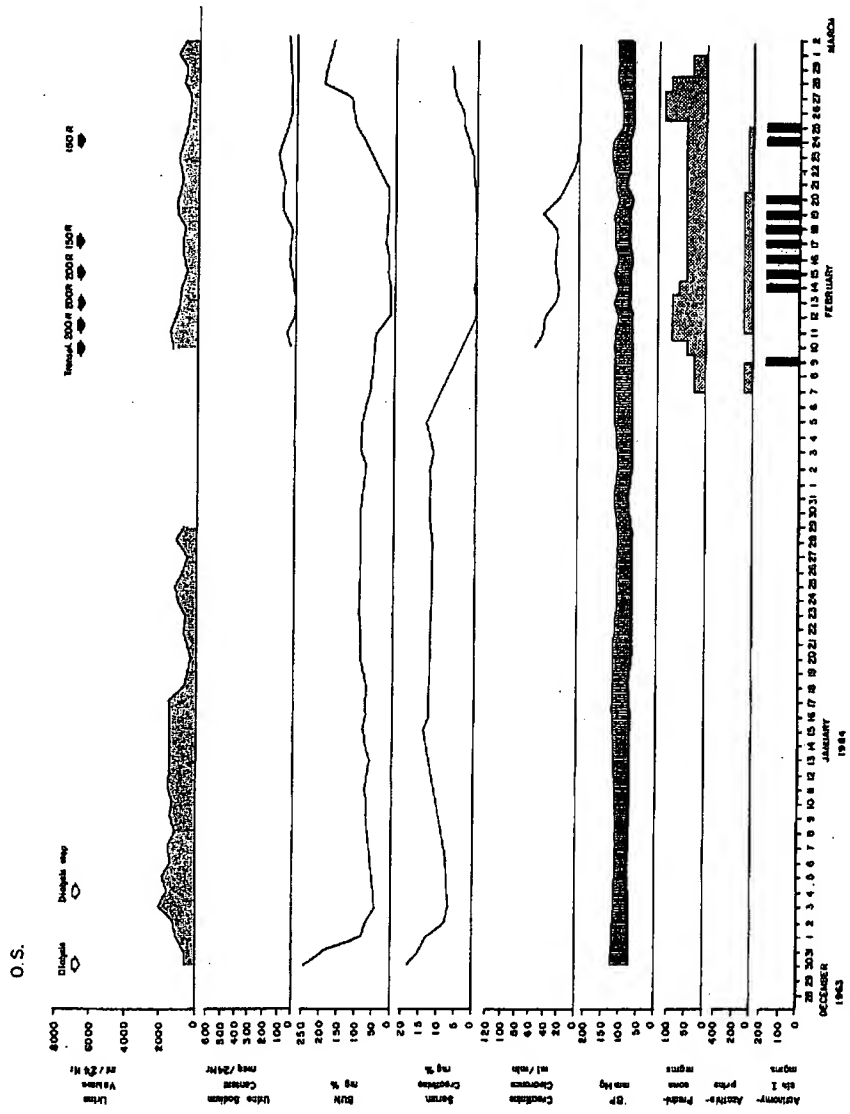

FIG. 30. Chart in Case 6 illustrating certain clinical features, laboratory studies, and drug treatment.

FIG. 31. Photomicrograph of transplanted kidneys in Case 6. Sections show hypercellularity of the glomerulus, interstitial edema, tubular degeneration and peri-vascular cellular infiltration.

from a pretransplant level of 1:32 to 1:1024. This gradually returned to pretransplant levels by day 30 and was below this at the time of death on day 63. Cytotoxicity tests show minimal and equivocal response on day 10. Other samples have not been tested as yet.

The patient in Case 2 showed a pretransplant natural anti-human A (A^h) titer of 1/8 which had decreased steadily to 1/2 by day 35. There was no natural anti-chimpanzee erythrocyte (Ch-rbc) titer before transplantation, nor did any appear later. Cytotoxicity tests through day nine were negative. This is the only patient that showed such a complete lack of response.

The patient in Case 3 showed a slight but significant rise in anti-O^{ch}rbc heterohemagglutinin titer following transplantation, but it did not appear until 23 days after transplantation in contrast to four other patients. This coincides in time with a delayed rejection crisis in this patient and may be correlated with it. There is other information, however, which contradicts this conclusion. The anti-A^{ch}rbc titer rose eight days after transplantation, in common with findings in the other patients. This did not change during the threatened rejection. Both titers had decreased by 40 days after transplantation despite retention of the functioning kidney. Cytotoxic antibody was present in high titer at day 23, and was still present, although waning, at day 36.

The patient in Case 4 showed two clearly separable responses in the hemagglutinine curve. First, immediately after transplantation of the incompatible type A kidney, both anti-A^{ch} and anti-$A_2{}^H$ titers dropped precipitously, and rebounded past pretransplant levels after its removal. That the same antibody is being measured in the two tests and that it was adsorbed by the trans-

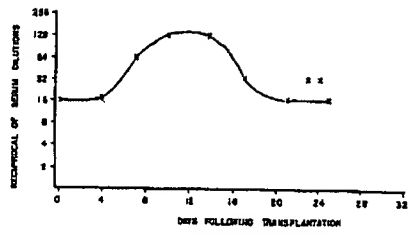

FIG. 32. Heterohemagglutinination studies in Case 6.

planted kidney is suggested by a) the failure of the kidney to function at any time, b) the reciprocal adsorption of the anti-A titer with H-rbc and ch-rbc and c) the adsorption of both the anti-A^{ch} and anti-$A_2{}^h$ titer by chimpanzee lymph node cells and acetone-dried splenic tissue. Second, after transplantation of a type O kidney there was a rise in the anti-Ch heterohemagglutinin titer not adsorbable by $A_2{}^H$-rbc nor by chimpanzee node lymphocytes. No cytotoxic antibody was found at day 7.

The patient in Case 5 showed an abrupt drop in natural anti-Ch rbc titer immediately after transplantation with a return to pretransplant levels by day five. Little significance can be attached to the other changes as too few serum samples were available. Cytotoxicity tests were negative at days one and 15.

The patient in Case 6 showed a rise in anti-A^{ch}rbc heterohemagglutinin from a pretransplant level of 1/16 to 1/128 by day 10 with a decline to normal by day 20. The loss in titer occurred despite reduction of immunosuppressive therapy and retention of the kidney for three days. Cytotoxic antibody was present the day after azathioprine and actinomycin were discontinued and remained at approximately the same level for at least nine days after removal of the kidney. Whether this antibody appeared earlier is not known, but it was not found on day 10 which marked the height of the HA response and the day that a clinical rejection pattern appeared.

It is our interpretation that the normal human anti-chimpanzee heterohemagglutinin is directed against an antigen present only on the erythrocyte and that the rise in titer following transplantation is a secondary response to a single, minimal antigenic exposure to red blood cells left in the kidney despite perfusion. The failure to adsorb this antibody either from patient's sera or from normal human serum with chimpanzee buffy coat leukocytes or lymph node cells is the main supporting evidence. Other evidence is the drop in this titer in all patients and in spite of retention of the kidney in the absence of immunosuppressive therapy in one patient. This response, then, reflects the ability of the patient to respond to a secondary stimulus and is an anamnestic response. Presumably if there were other pre-existing heteroimmune systems directed against nucleated cells or against kidney tissue specifically, they too would be capable of stimulation in the presence of immunosuppressive therapy. The stimulation of anti-A response by transplantation of a kidney from a type A donor to an incompatible recipient falls in this class and this result was seen in one patient. The total HA response was composed of a minor component meeting the criteria outlined above and a larger response directed against A^h-rbc, A^{ch}-rbc and chimpanzee type A lymphoid tissue.

The presence of cytotoxic antibody in some of the patients may, on the other hand, directly reflect a tissue immunity. Whether it is responsible for the clinical signs of rejection is not known, but its appearance may coincide with a rejection crisis rather than with the heterohemagglutinin response. Its appearance in only some patients and then not before day 10 suggest that it is not a secondary response. More results must be obtained to examine this important point further, as it is apparent that the immunosuppressive therapy used was not successful in preventing the secondary response to Ch-rbc.

Pathologic studies have revealed no consistent pattern. Sections of the grafts from patients in Cases 1 and 2, who survived for approximately two months, showed interstitial edema and tubular necrosis, perhaps related to terminal shock and sepsis. There were minimal changes in the glomeruli and no significant cellular infiltration.

The sections of kidneys from the patient described in Case 4 showed various changes. In the first transplant, in which kidneys were transplanted from a type A donor to a type O recipient, marked changes were seen, including interstitial cellular infiltration and edema, fibrinoid necrosis of blood vessels and fibrin thrombi in the glomeruli. In the second transplantation in Case 4, when kidneys from a group O donor were used, changes were less marked, with minimal cellular infiltration and normal glomeruli, but showing interstitial edema and tubular degenerative changes.

Pathologic studies of the transplant in Case 5 showed fibrinoid necrosis of blood vessels, tubular degeneration and moderate cellular infiltration. In Case 6, sections of the transplant showed increased cellularity of the glomerulus and moderate perivascular cellular infiltration.

Our tentative and preliminary over-all findings suggest that the heterografted kidney from chimpanzee to man may respond similarly to the homografted kidney from man to man. In a recent instance of renal homografting between sisters, threatened rejection occurred four days after transplantation, following a similar pattern to that seen in Case 1 reported here. There is similarity in time and degree of clinical and chemical changes seen in these two patients, in the initial response to transplantation, in the period of threatened rejection, and in reversal of rejection (Fig. 33).

Our further efforts will be directed toward defining the determinants of compatibility in matching of donor and recipient; toward adjusting of immunosuppressive measure to permit acceptance of renal heterografts; and toward studies of the long-term function of such grafts in man.

We would emphasize, however, that we regard this work as wholly experimental. Under these circumstances only the most stringent precautions will make such work justified and justifiable, and historic experience shows that the field of heterotransplantation may be abused flagrantly. The use of non-human kidneys in man removes the problems of the human donor, but the consequence of this exchange is increased difficulty imposed by cross-species transplantation. Whether heterografted kidneys will function often enough, well

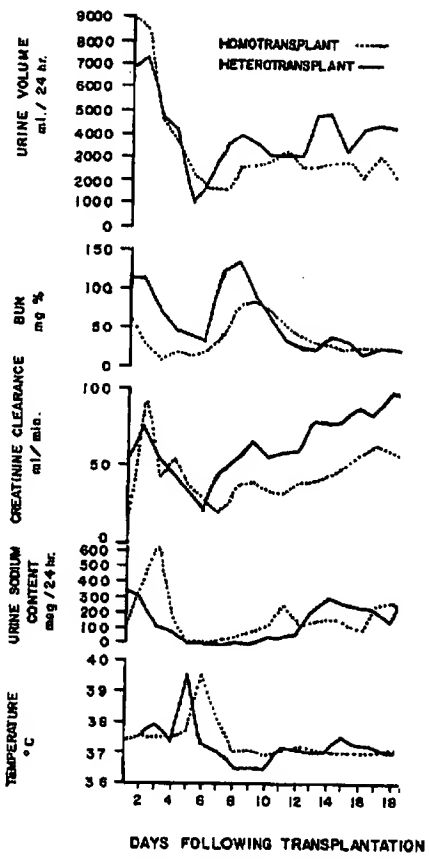

FIG. 33. Chart illustrating studies in two patients. The dotted line indicates studies in a patient receiving a homograft from her sister. The solid line indicates studies in the patient (Case 1) receiving an heterograft. Both patients underwent threatened rejection which was later reversed. These studies indicate certain similarities in time and degree of changes.

NAME _____ HOSPT _____ MEDICAL RECORD NUMBER _____

DAILY SUMMARY

DATE: ___/___/19___ (mo / day / year)

24 HOUR CLOCK ____ ____ ____ ____

Reports

Temperature (°C)

Yield = M

3. High

5. AM ( )

5. PM ( )

9. Comments ( )

Blood Pressure

		Systolic	Diastolic
High	D2B	( )	D3C ( )
Low	D4B	( )	D4C ( )
Comment:	D8	( )	

Intake-Output and Excretion

Intake

D. Blood Transfusions (ml) ( )

F 4 Q. Total 24-hour fluid intake (ml) ( )

L. Sodium (meq) ( )

N. Potassium (meq) ( )

F 4 Q. Diet ( )

24-Hour Urine Output (and concentrations)

F 5 A. Volume ( )

	Concentrat./L	24-Hr. Output
Sugar (mg)	F5D2 ( )	F5D5 ( )
Protein (mg)	F5F2 ( )	F5F5 ( )
Ammonia (mOSM)	F5J2 ( )	F5J5 ( )
Urea (mg)	F5L2 ( )	F5L5 ( )
Creatinine (mg)	F5N2 ( )	F5N5 ( )
Sodium (meq)	F5P2 ( )	F5P5 ( )
Potassium (meq)	F5Q2 ( )	F5Q5 ( )
Chlorides (meq)	F5R2 ( )	F5R5 ( )

F 5 2. Other (specify) ( )

3. Total 24-hour Output (ml) ( )

Weight (Kg)

F 7 E. AM ( )

G. PM ( )

Page 1 1076-010

FIG. 34. Sample page from data-processing form for heterografting studies.

enough and long enough to warrant their continued use remains unanswered, but the present study suggests to us that further work in this area is indicated.

As work in heterotransplantation is beginning there is an opportunity to devise systems of data-processing using newly developed biomedical computer systems. A system is now in operation in which data are transferred from the standard clinical charts or flow sheets to data-processing forms (Fig. 34). These data are then coded and stored. The information then may be retrieved in various forms. Complete data, in the narrative form, may be printed out (Fig. 35), giving a record of complete information on a daily basis. Additionally selected data may be retrieved and recorded in a sequential form (Fig. 36), permitting analysis of trends in a single patient or among several patients. Such data collected from centers engaged in heterotransplantation will be processed and returned to the investigators, with summaries of all current work in heterotransplantation. Furthermore, all data from the various centers will be available to each investigator. Perhaps this rapid dissemination of data will aid in reducing the repetition of mistakes.

Summary

The use of chimpanzee kidneys in renal heterotransplantation into man is under investigation.

Recipients, all patients in terminal uremia who have been dialyzed, received grafts from donors selected on the bases of blood groups and body size.

Immunosuppressive measure included azathioprine, actinomycin C, steroids and local x-radiation.

Maximal functional survival of the transplant has been six months. Sepsis has proved to be a more frequent and lethal complication than rejection.

Immunologic response has been followed by heterohemagglutin titers and cytotoxicity studies. Pathologic studies have shown in general, tubular degeneration and interstitial edema without marked cellular infiltration or vascular changes.

The highly experimental nature of the study is emphasized, and caution is urged in clinical heterotransplantation.

In transplantation, a method for international cooperation in data-processing and data-sharing, using biomedical computers, is proposed.

Addendum

Six months after receiving an heterotransplant, the patient described in Case 3 is asymptomatic and is followed as an outpatient. Her renal function is normal and her white blood cell count ranges between 5,000 and 6,000. Immunosuppressive medications are azathioprine 75 mg. and prednisone 30 mg. daily.

```
I.D.NUMBER MCH#F93108853                                    PAGE  9

     AM - 37.8
     PM - 37

BLOOD PRESSURE
  HIGH
     SYSTOLIC - 140
     DIASTOLIC - 90
  LOW
     SYSTOLIC - 110
     DIASTOLIC - 70

INTAKE-OUTPUT AND EXCRETION
  INTAKE
     TOTAL 24-HOUR FLUID INTAKE - ML - 4630
     DIET - BLAND
  24-HOUR URINE OUTPUT - AND CONCENTRATIONS
     VOLUME - 3700
     SODIUM - MEQ
          24-HOUR OUTPUT - 320
     POTASSIUM - MEQ
          24-HOUR OUTPUT - 104
  TOTAL 24-HOUR OUTPUT - ML - 3700
  WEIGHT - KG
     AM - 49

HEMATOLOGY
  HEMATOCRIT - 29
  WBC - 5000
  PLATELETS - 158000

BLOOD CHEMISTRY
  ELECTROLYTES
     SODIUM - MEQ/L - 147
     POTASSIUM - MEQ/L - 4.8
     CHLORIDE - MEQ/L - 101
     CARBON DIOXIDE - MEQ/L - 26.
  NON-ELECTROLYTES - MG PER.
     BUN - 9
     CREATININE - 0.6

UROANALYSIS
  MICROSCOPIC - ABNORMAL QUANTITY PRESENT
     CELLS

TREATMENT

     DRUGS ORDERED
     PREDNISONE
       40 MG
     IMURAN
       200 MG
     ANTIBIOTIC - CHLOROMYCETIN 2 GMS PENICILLIN 2,400,000U
```

FIG. 35. Print-out form of a narrative type derived from data-processing forms.

```
IC NUM-MCH#F1931088343        DATE OF BIRTH-16- 2-40   SEX-F        PAGE  1

                    24-HOUR URINE OUTPUT          ELECTROLYTES          NON-ELECTROLYTES      CREAT.
DATE                                                                    BUN     CREAT.    CLEARANCE
DY MO YR   TIME   VOLUME   NA+    K+     AA+     K+     CL-    CO2
23 01 64   0080    4008    N/A    N/A     137    5.7     93     18      116      21       N/A
14 01 64   0080    6485     353     71     143    4.2    108     26       47     2.7       N/A
15 01 64   0000    4320     347     196    144    3.7    105     30       12     0.9        99
16 01 64   0000    3970     352     184    149    3.4    100     29        9     0.9        95
17 01 64   0000    3500     346     180    143    2.7    104     29        9       1       N/A
18 01 64   0000    3700     328     185    146    3.4    102     29        5     0.8        94
19 01 64   0080    3700     320     184    147    4.8    101     26.       9     0.6       N/A
20 01 64   0000    4400     299     184    141    3.5     98     28        5     0.4        90
21 01 64   0000    3120     182     178    141    3.4     94     30        9     0.6        62
22 01 64   0000    2080     290     254    140    4.2     97     29       9.0    0.3        75
```

FIG. 36. Sample of selected daily values in one patient retrieved from data processed through the bio-medical computer center and printed in sequential form.

Acknowledgments

The work on primates was made possible by the efforts of Dr. Kenneth Burns and Dr. Arthur Riopelle. Anesthesia was supervised by Dr. John Adriani and the anesthesiologist participating in the primate work was Dr. David Scully. Radiotherapy was supervised by Dr. J. V. Schlosser. Renograms were made by Dr. Jack Mobley. We are indebted to the following for donations of chimpanzees: Major C. H. Kratochvil, USAF M.C., Commander, 6571st Aeromedical Research Laboratory, Holloman AFB, New Mexico; Major J. D. Marshall, Walter Reed Army Medical Center, Washington, D.C.; Mr. George Douglas, New Orleans Zoological Garden, New Orleans, Louisiana; and the Delta Regional Research Primate Center.

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Discussion

Dr. Joseph Murray (Boston): Dr. Reemtsma's persistence in the study of heterotransplantation, although as yet not of clinical usefulness, has opened up this phase of transplantation biology for further experimental investigation. None of us one year ago would have guessed that any primate graft would have survived for 12 weeks. Certainly no one would have expected that any would have had a reversal of a rejection process, as has been so beautifully demonstrated by Dr. Reemtsma.

Primate grafts may in the future serve as a potential source of donor organs, and this possibility alone warrants further laboratory study in their use.

It is a tribute to Dr. Reemtsma's group that their initial failure did not dissuade them, as it has so many others in the past 50 years. Their persistence reflects an open-minded attitude so necessary for the accumulation of valid scientific data.

Dr. Reemtsma mentioned that we have established a registry of human kidney homotransplants under the aegis of the National Research Council and with the help of the Public Health Service. This registry is open to everybody performing or contemplating human kidney homotransplantation. We will send data sheets to anyone requesting them. All data will be tabulated, processed, and programmed on a semi-annual basis, and published in the journal *Transplantation* and all information will be available on request to all at any time.

Discussion

Dr. David M. Hume (Richmond): It seems to me that it may be worth while to continue doing some experiments with heterografting in man, although I must say I was among those who thought it was pretty foolish at the start, because it did not seem likely that it was going to work clinically, and, of course, it still has not proved itself in this regard.

Nonetheless, there are some types of grafts which will have to come from a source other than man himself, and the heterograft, if it works, may provide this source.

I think Dr. Reemtsma and his group have done a careful study of renal heterografting in man and this is something which it might be well for them to continue doing. I do not think it is anything that can achieve general acceptance at the moment, even experimentally.

We did one chimpanzee transplant in man some time ago, and ran into one difficulty which I rise to mention at the present time: The patient had not been as well prepared for surgery as some of our subsequent or previous patients. He was still rather wet. He had 4+ ankle edema, 3,000 to 4,000 cc. of ascitic fluid, bilateral moist rales, and an extremely puffy face.

We transplanted him from a 130-pound chimp, using both kidneys, which probably we should not have done. The total kidney mass was greater than a single human kidney, about 340 Gm. The kidneys functioned at once. They began to produce urine extremely vigorously and put out 54,000 cc. in the first 24 hours.

This posed a massive problem in fluid-electrolyte balance. We began by holding back and giving the patient only half as much fluid intake as he put out, even though we fell far behind within a matter of six hours. The patient who had been extremely edematous, as I have described, lost all of his ankle edema completely. He lost all of his ascites, and his face, which had been swollen, became sunken, and by the next morning he was dehydrated. His sodium and potassium were extremely hard to manage. Chimps take a diet which is high in potassium, and they have kidneys which handle it much more easily than man. His potassium fell to 2.0 mEq./L, and we had to give him something like 600 mEq. of potassium to keep up.

The patient suffered a stroke the following morning, and lived for about three days, at which time he had a coronary and died.

There are some severe problems with this type of graft, apart from the immunological ones, and I certainly do not believe it is anything that should be widely applied to patients at this time. We do not plan any more in the future, but we plan to watch Dr. Reemtsma carefully.

Dr. T. E. Starzl (Denver): Dr. Waddell regrets being unable to attend this meeting, but he asked me to speak of the six baboon heterografts which were done in Denver after considerable helpful guidance from the New Orleans group and in active collaboration with Dr. Claude Hitchcock of Minneapolis.

One of the basic objectives in this study was to compare the behavior of the baboon heterograft with that of the chimpanzee, because the baboon is far more plentiful, there being about 500 for every chimp, and far less expensive, the cost of a baboon being only about $250.00.

Good renal excretion was obtained from all six heterografts. However, cross-comparison with Dr. Reemtsma's data suggests that the baboon does not provide as good immediate or sustained function as the chimpanzee. The pathologic specimens from our cases have been submitted to a referee. Dr. Reemtsma has submitted some of his autopsy material to the same referee, and it would appear that the pathologic changes in the baboon are also more severe than with the chimp.

The six patients who we operated upon were turned down for standard homotransplantation for various medical contra-indications. Four of the six patients are dead. Two of them are still living, but only by virtue of a secondarily performed homotransplantation, and it is of interest that the homografts functioned well despite the prior presence of the heterografts much like the experience which has been described with serial homografts.

(Slide) The first slide shows one of the heterografts. This one showed a very aggressive cellular reaction after about 25 days, although the heterograft had grossly not seemed to be severely damaged.

(Slide) The next slide shows a heterograft which was removed after 40 days, and this is the type of pathologic finding which characterized all of the other five. One can see the many infarcted areas on the surface of the kidney, and also the swollen appearance of these organs.

Because of our experience with this group, it is our opinion that baboon heterografts cannot work on a long-term basis at the present time. The results we have obtained are less encouraging in terms of function and pathologic tissue injury than have been found in Dr. Reemtsma's chimpanzees. We have, therefore, abandoned the use of the baboon heterografts.

Dr. Willard E. Goodwin (Los Angeles): At UCLA in Dr. Longmire's department we have been interested in heterotransplantation of the kidney for several years. However, we thought that it was a study for the laboratory up till now. We have done dog to sheep, sheep to dog, dog to monkey, human to dog three times, human to monkey once, monkey to monkey of a different species and human to chimpanzee transplants. All of these have been rejected, usually with an accelerated pattern. In the dog to sheep and

sheep to dog it happened while were were still watching it. The monkey to monkey of a different species was the most promising. This kidney survived for two months.

Human to chimpanzee heterotransplantation which we have done only once lasted a week, and the animal died probably not of rejection, but rejection was taking place in the kidney.

We have the greatest admiration for experiments of this sort and for the work that has been done and is being done in New Orleans and Denver. I would like to say that it may be that the most important observation that comes out of this is that transplanted kidney from a primate to man is capable of clearing vast quantities of fluid and acting as a crutch to the future in acute situations. Maybe this would be a cheaper and more appropriate way of handling this situation than an artificial kidney when you are dealing with a given limited time period in which you expect the patient to recover.

DR. KEITH REEMTSMA (Closing): I should like to acknowledge with gratitude the help we have received from the Boston, Richmond and Denver groups, particularly in the area of immunosuppression.

The other members of our group join me in expressing our appreciation to the Dean of our medical school, Dr. Charles C. Sprague, and to the director of anesthesiology at Charity Hospital, Dr. John Adriani, a member of this association. They have supported our work strongly from the outset.

Finally, I wish to thank the American Surgical Association for the privilege of presenting this work. Historically the field of heterotransplantation has garnered a rather unsavory reputation because of outright abuses and extravagant claims. The inclusion of this report on the program of the Association will aid immeasurably in restoring heterotransplantation to the position which we believe it deserves: an appropriate field for scientific investigation.

Immunosuppression after Experimental and Clinical Homotransplantation of the Liver *

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THE FATE of whole organ liver homografts after transplantation to untreated canine recipients is well known.[3, 9, 28, 29, 26, 34, 39, 41] For several days after operation, the transferred tissue is life-sustaining, but after this there is rapid functional failure. The histologic abnormalities in the rejected liver consist of infiltrates of mononuclear cells which tend to be concentrated in the periportal areas, a necrotizing arteriolitis, and dissolution of hepatic parenchymal cells with retention of a relatively normal reticulum.

In the present report, an attempt will be made to add an additional dimension by describing the behavior of canine and human hepatic homografts in recipients treated with immunosuppressive agents. Under these circumstances, a modified rejection was observed in many instances despite the absence of significant cellular invasion of the homograft. In addition, it was noted that severe hepatocyte injury often occurred in livers in which the duct system was selectively preserved or even hyperplastic. Finally, several previously unrecorded observations will be documented regarding more esoteric biochemical alterations in patients after hepatic homotransplantation. These include serial determinations of plasma or serum immunoglobulins, haptoglobins, amino acids, pyruvates, and lactates.

Methods

Types of homotransplantation. Orthotopic homotransplantation was carried out in 25 dogs, after removal of the animal's own liver.[40] The reconstructed blood supply to the revascularized homograft was essentially normal.[40] The time for transfer and complete revascularization of the cooled organ averaged 70 minutes. The effects of ischemia were minimized by perfusion of the liver with cold (10–15° C.) lactated Ringer's solution prior to its removal.[40] Internal biliary drainage was provided with a cholecystjejunostomy or cholecystoduodenostomy. Splenectomy was performed. Eleven animals died during or within three days following operation. These failures were considered to be technical,[42] and are not considered in the pathologic analysis (Table 1).

In 15 dogs, an auxiliary liver was placed in the right paravertebral gutter (Fig. 1) using a modification[42] of the method of Welch and his associates,[9, 47] revascularizing the portal vein from the terminal inferior vena cava and the hepatic artery

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EXHIBIT 6

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ARTICLES

Baboon-to-human liver transplantation

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Our ability to control both the cellular and humoral components of xenograft rejection in laboratory experiments, together with an organ shortage that has placed limits on clinical transplantation services, prompted us to undertake a liver transplantation from a baboon to a 35-year-old man with B virus-associated chronic active hepatitis and human immunodeficiency virus infection.

Liver replacement was performed according to conventional surgical techniques. Immunosuppression was with the FK 506-prednisone-prostaglandin regimen used routinely for hepatic allotransplantation, to which a daily non-myelotoxic dose of cyclophosphamide was added. During 70 days of survival, there was little evidence of hepatic rejection by biochemical monitoring or histopathological examination. Products of hepatic synthesis, including clotting factors, became those of the baboon liver with no obvious adverse effects. Death followed a cerebral and subarachnoid haemorrhage that was caused by an angioinvasive aspergillus infection. However, the underlying cause of death was widespread biliary sludge that formed in the biliary tree despite a seemingly satisfactory choledochojejunostomy. During life and in necropsy samples, there was evidence of the chimerism that we believe is integral to the acceptance of both xenografts and allografts.

Our experience has shown the feasibility of controlling the rejection of the baboon liver xenograft in a human recipient. The biliary stasis that was the beginning of lethal infectious complications may be correctable by modifications of surgical technique. In further trials, the error of over-immunosuppression should be avoidable.

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Introduction

Previous attempts to transplant seven baboon kidneys[1,2] and two hearts[3,4] resulted in graft loss or patient death between 0 and 60 days after transplantation. A common difficulty was uncontrolled cellular rejection, together with antibody-mediated occlusive endotheliolitis of graft microvasculature and parenchymal necrosis.[4,5] Recent laboratory investigations have shown that the presumably humoral component of xenograft rejection could be diminished by a short course of antimetabolite therapy, such as cyclophosphamide, which targeted the B-cell proliferative response.[6-8] By overcoming this antibody barrier, the value of maintenance therapy with T-cell-directed immunosuppressants was unmasked.[6-8]

We now describe a baboon-to-human liver xenotransplantation in which FK 506 and cyclophosphamide were given as immunosuppressants, together with prednisone and prostaglandin, both of which help to mitigate preformed antigraft antibody syndromes and cellular rejection.[9,10]

Patient and methods

Recipient history

A 35-year-old white male had a history of abnormal liver function tests since 1984 with recurrent bleeding from oesophageal varices and haemorrhoids which began 2 years later. Hepatitis B virus (HBV) and human immunodeficiency virus (HIV) had been diagnosed in 1987. When his spleen was ruptured and removed after a motorcycle accident in 1989, his prothrombin time (PT) was

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TABLE I—DONOR AND RECIPIENT BIOCHEMISTRY

—	Albumin (g/L)	Total protein (g/L)	Cholesterol (mmol/L)	PT (s)	Alkaline phosphatase (IU/L)	Uric acid (μmol/L)
Donor baboon	18	48	1·03	10·9	387	<30
Patient pre-op	20	63	2·33	16·3	158	481·8
Patient 5 days post-op	20	52	1·16	12·5	452	208·2
Patient 45 days post-op	19	40	1·71	11·5	2812	<30

PT, prothrombin time.

15·7 s, aspartate aminotransferase (AST) 105 IU/L, alanine aminotransferase (ALT) 73 IU/L, albumin 2·7 g/L, and total bilirubin 47·9 μmol/L. Macronodular cirrhosis of the liver was noted at the time of splenectomy, and a biopsy specimen confirmed the clinical diagnosis of chronic active hepatitis. After being refused liver transplantation elsewhere, he came to Pittsburgh in January, 1992, with jaundice, spider naevi, ascites, peripheral oedema, episodic encephalopathy, and deteriorating hepatic function. Hepatitis A, C, and delta were negative. Hepatitis B surface antigen (HBsAg) was positive and antibodies to hepatitis B core antigen were also present; e antigen was negative. There was serological evidence of previous infection with Epstein-Barr virus (EBV), cytomegalovirus (CMV), and herpes simplex virus (HSV).

His clinical condition worsened between January and May, 1992, and he eventually required continuous hospital care. Because the baboon liver was thought to be resistant to HBV infection (J. Hoofnagle, National Institutes of Health, personal communication), baboon-to-human liver xenotransplantation for HBV hepatitis was already under discussion by the Institutional Review Board of the University of Pittsburgh and members of US government agencies. Although the HIV carrier state was an undesirable factor, the patient was accepted into the HBV xenotransplantation protocol because of his urgent clinical status. Prophylactic antiviral therapy with ganciclovir was started, but hyperimmune anti-B virus globulin was not given.

Donor surveillance

The 15-year-old male baboon (*Papio cynocephalus*), who had the same A blood group as the recipient, was obtained for organ donation from the Southwest Foundation for Research and Education, San Antonio, Texas. Retrovirus antibody screen revealed the animal to be negative for Simian T-lymphotropic virus, human T-cell leukaemia virus 1 and 2, Simian immunodeficiency virus, Simian retrovirus 1, 2, and 5, and HIV 1 and 2. He was antibody positive for foamy virus, and had evidence of previous infection with EBV, CMV, Simian agent 8, and Varicella-Zoster virus. Antibodies directed against herpes simplex virus and herpes B virus were not present. In addition, there was no evidence of infection with HBV, HAV, HCV, Marburg virus, encephalomyocarditis virus, lymphochoriomeningitis virus, and haemorrhagic fever virus.

Donor biochemical tests that fell outside normal human ranges included alkaline phosphatase 387 IU/L, serum uric acid <0·5 mg/dL, serum cholesterol 1·03 mmol/L and serum albumin 18 g/L (table 1). These values are normal for the baboon.

Transplantation and postoperative course

Liver replacement was done on June 28, 1992, by conventional techniques including venovenous bypass.[12] The 600 g liver from the 25·8 kg baboon donor was preserved with University of Wisconsin solution for 137 min of cold ischaemia. It was inserted by the piggy-back technique into the 70 kg recipient whose excised liver weighed 1750 g. Xenograft venous outflow was through the transplant suprahepatic vena cava into a cuff of the left and middle veins of the excised native liver, which drained into the intact retrohepatic inferior vena cava of the host. The xenograft coeliac axis and portal vein were anastomosed to the recipient common hepatic artery and left portal vein, respectively, after oversewing the right portal branch. Biliary reconstruction was with a roux-en-Y choledochojejunostomy. 20 units of blood were given during the 11 h operation. Generalised wound bleeding secondary to pre-existing coagulopathy (PT 16·3 s) diminished substantially after the xenograft was revascularised.

FK 506, prednisone, and prostaglandin are given routinely after liver allotransplantation (fig 1).[13] Except for higher doses of FK 506 given during the first 2 postoperative weeks, the doses of all 3 drugs were within standard therapeutic ranges. In addition, non-myelotoxic doses (25 to 175 mg/day) of cyclophosphamide were begun 2 days preoperatively and continued for 55 days (fig 1). All four drugs were changed from the intravenous to the oral route when diet was resumed. Doses of intravenous prostaglandin were 0·4 to 1·0 μg/kg per hour PGE_1 and for the oral form were 6 μg/kg per day PGE_2 (misoprostil). From days 12 to 21, prednisone was stopped except for a 1 g bolus of methylprednisolone.

Postoperative investigations

Biochemical studies—Standard liver and renal function tests were repeated daily. In addition, the arterial ketone body ratio, which estimates hepatic mitochondrial integrity and liver energy charge,[14] was monitored daily for the first 30 days. Attention was paid especially to tests whose normal ranges were strikingly divergent in the baboon and human: protein electrophoresis, albumin, alkaline phosphatase, uric acid, and cholesterol.

Immunological studies—Complement-dependent cytotoxicity was measured in pretransplant and post-transplant patient serum samples that were tested against lymphocytes from either the donor baboon or a panel of third-party human donors. Serum samples were tested with and without dithiothreitol (DTT) treatment, which inactivates IgM, thus permitting detection of IgG.[15] Donor

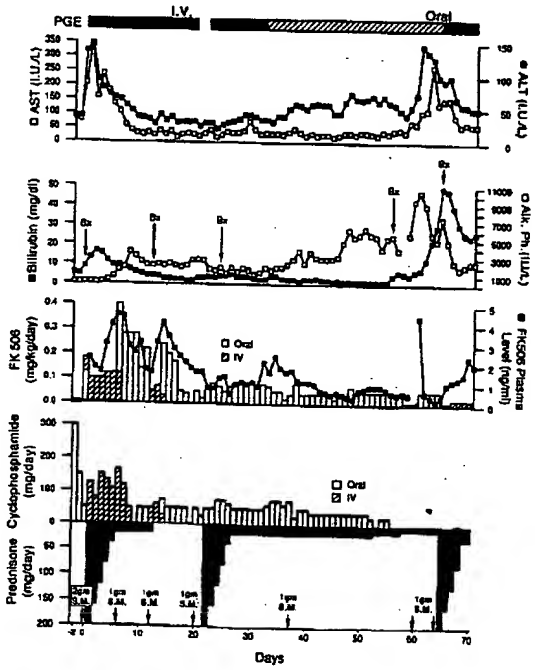

Fig 1—Clinical course after baboon liver transplant.

SM, Solumedrol (methylprednisolone); PGE, prostaglandin E; Bx, biopsy; AST, aspartate aminotransferase; ALT, alanine aminotransferase; Alk Ph, alkaline phosphatase.

TABLE II—POST-TRANSPLANT INFECTIOUS COMPLICATIONS

Postoperative day	Infection	Organism	Treatment
7	Wound infection and bacteraemia	Staphylococcus	Vancomycin and debridement
27	Bacteraemia	Staphylococcus	Vancomycin
29	Oesophagitis	Candida albicans	Amphotericin
30	Viraemia	Cytomegalovirus	Ganciclovir
42	Oesophagitis and viraemia	Cytomegalovirus	Ganciclovir
55	Bacteraemia	Enterococcus faecalis	Vancomycin
56	Bacteraemia	Staphylococcus	Vancomycin
61	Sepsis	Presumed cholangitis after percutaneous transhepatic cholangiogram	Amikacin and imipenem/cilastin
68	Sputum	Aspergillus flavus	Amphotericin
70	Death	Disseminated aspergillus (at necropsy)	

baboon lymphocytes were isolated at the time of transplantation from lymph nodes and spleen, and were cryopreserved in tissue culture media supplemented with 20% pooled baboon sera. Dilutions of patient sera were made to 1 in 512 for pre-transplant samples and 1 in 32 for post-transplant sera. Rabbit complement for crossmatch testing was absorbed with baboon red cells. Pooled human sera and pooled baboon sera were used for positive and negative crossmatch controls, respectively. Daily samples were collected from the patient, batched and tested every three days in the first month post-transplant.

For mitogen response assays, peripheral blood lymphocytes from heparinised blood samples of the xenograft recipient were isolated by a Ficoll-Hypaque gradient and were resuspended at a concentration of 5×10^4 cells per well for 72 h at 37°C with either concanavalin A (ConA) (4 μg/ml) or phytohaemagglutinin (PHA) (10 μg/ml).

Unidirectional mixed lymphocyte reaction (MLR) cultures were set up with 5×10^4 responder (patient) and 5×10^4 irradiated baboon lymphocytes (donor) or 5×10^4 irradiated third-party human lymphocytes (allocontrol) in 200 μl tissue culture medium for 6 days. Cryopreserved baboon lymphocytes were thawed and prepared in 5% baboon serum.

Total complement activity (CH100) was quantitated by an agarose-gel method that measured lysis by test sera of sheep red blood cells (Kallestad, Austin, Texas).[16] Antigen-antibody complexes were detected on stained agarose gels after routine zone electrophoresis.[17]

Chimerism was determined by polymerase chain reaction amplification of the baboon chorionic gonadotropin gene. Primers (CCACCCCATGGTCTCCGTTC and GAACGGGGTGCC-TGCTCC) were selected from the middle exon of the beta subunit of baboon chorionic gonadotrophin such that they did not amplify the corresponding human sequence.[18] The sensitivity and specificity of the method was determined by amplification of baboon DNA serially diluted with human DNA. The quantity of baboon DNA present in the various tissues was estimated from an autoradiogram of a Southern blot of the PCR products after hybridisation with a radiolabelled probe prepared from the baboon gene.

Histopathological studies—Wedge or needle biopsy specimens of the xenograft liver were obtained at operation and on days 12, 24, 55, and 64 post-transplantation. One part was frozen in Optimum Cold Temperature for immunofluorescent studies (IF) and the remainder was fixed in formalin. Anti-baboon antibodies were sought by indirect IF microscopy (tissue crossmatch) with the recipient's serum as the primary antibody, followed by fluorescein-labelled anti-human IgG and IgM as the secondary immunoreactants. Frozen tissue was also studied with direct IF using anti-human IgG, IgA, IgM, C1q, C3, C4, fibrinogen, alpha-2-macroglobulin, and albumin.

Fig 2—Cholangiogram on day 61.

The ducts are much larger than at the time of transplantation and the sharp cut off of a major duct (arrow) was probably because of sludge found at necropsy.

Phenotypic analyses of the cellular infiltrates with routine indirect avidin-biotin complex technique were performed on paraffin-embedded tissues with antibodies directed at T (L60, UCHL-1, OPD4 [CD4]), B (L26, IgG, IgM), and NK (Leu-7) cells, and macrophages and neutrophils (lysozyme). We adopted the same technique to monitor HBV infection (anti-HBsAg and anti-HBcAg) and proliferative activity (anti-proliferating cell nuclear antigen).

Results

Clinical course

After transplantation, our patient, who had lapsed into stage 3 coma preoperatively, woke promptly and was extubated after 17 h. He was eating and walking within 5 days. For the first two postoperative weeks, human albumin 50–75 g/day was given intravenously. Albumin was then administered only to cover losses from dialysis procedures or during plasmapheresis for haemoglobinaemia during the terminal phase of his postoperative course. The liver was 600 g at the time of transplantation and regenerated to a computed tomographic (CT) scan estimated size of 1074 g after one week and 1555 g by day 24. Although he was released from intensive care after one month, he developed several infections (table II) that necessitated treatment with nephrotoxic antibodies. The most disabling of these was mixed CMV and candida oesophagitis and duodenitis, which were suspected to be the cause of recurrent gastrointestinal haemorrhages from days 27–39 and which required 14 units of transfused blood. *Staphylococcus aureus* was cultured from the blood on postoperative days 6, 26, and

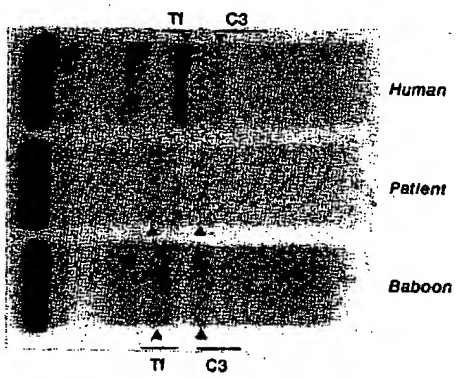

Fig 3—Protein electrophoresis of normal human, baboon donor, and patient serum.

C3, complement; Tf, transferrin.

55; *Enterococcus faecalis* was found cultured on day 55. On day 65, aspergillus was cultured from a tracheal aspirate.

Other complications included renal failure and dialysis dependence beginning on day 21, which probably resulted from multiple drug toxicity (FK 506, amphotericin, ganciclovir, and possibly vancomycin) and a right haemothorax from a liver biopsy on day 24. Despite these difficulties, the patient was afebrile and otherwise well until day 55 when he was readmitted to intensive care after jaundice recurred. Angiography on day 59 showed normal hepatic vascular anatomy; a transhepatic cholangiogram on day 61 was read as normal (fig 2). 1 h after cholangiography, he became hypotensive with rigors; he required intubation. There was evidence of disseminated intravascular coagulation and haemolysis with a fall in platelet count from 115 000 to 29 000 × 10^9/L, an increased free plasma haemoglobin of 87·5 mg/dl (normal <3·0), undetectable haptoglobin, and a rise in bilirubin from 212 mmol/L to 851·6 mmol/L during the next 48 h.

From days 65 to 70, the patient had 5 plasmaphereses that reduced serum bilirubin. On day 70, while being weaned from the ventilator, there was a sudden loss of higher nervous system function. A CT scan showed a massive subarachnoid haemorrhage and 6 h later he was declared brain dead.

Biochemical studies

The arterial ketone body ratio rose from 0·3 (low) during wound closure to >1·0 at 3 days and beyond, indicating excellent hepatic energy charge. Tests of liver injury (aminotransferase concentrations) were increased to a maximum of 250 IU/L during the first few days post-transplantation and after the cholangiogram. Bilirubin concentrations were also normal throughout most of the post-operative course (fig 1); however, alkaline phosphatase was increased from the end of the the first week onward.

Prothrombin time and other indices of hepatic synthetic function were similar to those measured preoperatively. The relative hypoalbuminaemia was well tolerated by the patient; he had no evidence of fluid retention at any time. Serum protein electrophoresis in the recipient showed that other protein fractions of hepatic origin promptly reverted to those of the baboon (fig 3). Plasma ammonia concentration was 139 mmol/L preoperatively, and fell to below 50 mmol/L after the first week (normal <50). Serum uric acid (normally <30 mmol/L in the baboon) fell from 481·8 mmol/L in our patient into the expected baboon range. A similar but less dramatic transition was seen with serum cholesterol (table I).

Immunological and viral studies

Preoperatively the recipient's serum samples had complement-dependent lymphotoxic antibody activity against 73% to 89% of a 45 cell panel of HLA-typed donors and against lymphocytes from 7 different baboons including the eventual donor. After DTT treatment, the 73–89% panel-reactive antibody before transplantation became 0%, and the patient's positive serum crossmatch with all 7 baboon donors became weak-positive. All serum samples post-transplantation were crossmatch negative after DTT treatment. Daily serum samples tested for donor-specific crossmatch without DTT showed minimum change in titres ranging from 1 in 8 to 1 in 16 throughout the first month post-transplant.

The total white blood cell count preoperatively was 13 × 10^9/L with 6·2 × 10^9/L lymphocytes (28% CD4, 58% CD8; ratio 0·48). After transplantation, the total number of white blood cells remained constant but the number of lymphocytes fell to below 0·6 × 10^9/L. Pretransplantation, he had a normal immune response to several stimuli, including mitogens (PHA and Con A), alloantigens, and other donor-baboon lymphocytes (table III). Postoperatively, our patient's lymphocytes failed to respond to allo or xeno antigens and the only significant proliferation took place with PHA.

The total complement (CH100) fell from normal preoperatively to below the limit of detection (<21 U/mL) for the first 8 postoperative days during which circulating antigen-antibody complexes were present (table IV). Thereafter, the CH100 ranged from slightly depressed to normal at the same time as complexes were detectable. The results were similar to those observed by Manez et al[16] in successfully treated recipients of lymphocytotoxic-crossmatch-positive allografts. With the return of CH100, C3, which is thought to be synthesised mainly in the liver,[19] was slightly depressed while C4 and C5 were normal. C4 is also thought to be mainly hepatic in origin.[20]

TABLE III—IN-VITRO TESTS OF IMMUNE REACTIVITY

Time (wks)	WBC (× 10^9/L)		Proliferative responses (net cpm)			
	Total	Lymphocytes	PHA	Con A	Xeno MLR	Allo MLR
Pre-operative	13	6·2	90,263	13,371	44,794	74,290
1	10·5	0·5	8,591	436	112	413
2	5·9	0·3	384	90	100	250
4	10·2	0·4	16,788	92	121	62
6	6·9	0·3	1,963	81	59	48
8	5·1	0·6	37,945	4,606	448	156
9	8·4	0·9	1,248	90	120	127

Cpm, counts per minute.

TABLE IV—TOTAL SERUM COMPLEMENT (CH100), COMPLEMENT COMPONENTS (U/mL), AND IMMUNE COMPLEXES (IC)

Days postop	CH100 (>60)	C3 (83–177)	C4 15–45)	C5 (6–20)	IC
1	<21	..	..	..	+
2	<21	..	..	..	+
4	<21	..	..	..	+
6	<21	..	..	..	
8	<21	..	..	..	+
9	<21	..	..	..	−
11	21	..	..	..	−
14	43	..	..	..	−
17	55	64	17	20	−
23	66	59	15	17	−
26	61	51	15	11	+(low)
28	44	40	13	14	−
33	55	58	14	15	+(low)
64	55	..	..	..	−

Baboon DNA was found in the patient's heart, lung, kidney, and two lymph nodes obtained at necropsy (fig 4). The blood samples collected during life also were positive. In some tissues, baboon and human DNA were found in a ratio of 1 in 1000 whereas in others, such as lymph nodes, the ratio was close to 1 in 10 000 (fig 4).

HIV status did not change significantly after transplantation. Cell dilution method for quantification of HIV-infected cells showed 66 infected cells per million peripheral blood mononuclear cells (PBMC) on days 2 and 17, with 16 infected cells per million PBMC on post-transplant days 31 and 59 (courtesy of Dr Phalguni Gupta, Graduate School of Public Health, University of Pittsburgh).

Pathological studies

Tissue crossmatch of the xenograft before revascularisation with pretransplant recipient serum showed a diffuse non-specific staining pattern with anti-IgG. Anti-IgM revealed faint portal vein staining, focal sinusoidal cell positivity, and distinct nuclear staining consistent with the preformed antibody detected on conventional crossmatch.

The 4 h post-perfusion biopsy revealed Kupffer cell hypertrophy, sinusoidal neutrophilia, and small platelet aggregates in the sinusoids and portal and hepatic veins, without hepatocyte necrosis or inflammatory or necrotising vasculitis. Direct IF showed diffuse sinusoidal fibrinogen (IgG > IgA > IgM) with linear-granular sinusoidal C1q,

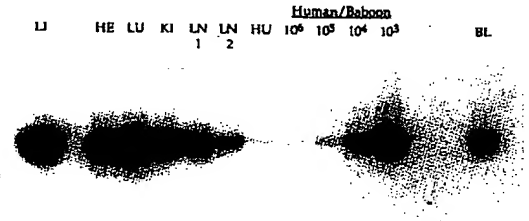

Fig 4—PCR amplification of baboon-specific DNA from recipient tissues.

Lane LI is the PCR product from baboon liver and contains only 1% of PCR reaction to avoid overwhelming the other lanes. HE, heart; LU, lung; KI, kidney. LN1 and LN2, lymph nodes 1 and 2. For semiquantitation, either human (HU) or baboon DNA was serially diluted into human DNA at the indicated ratios and tested, showing a baboon DNA concentration of approximately 0·1% in most specimens. Blood (BL) was obtained 35 days post-transplantation and all other samples were taken at necropsy (day 70).

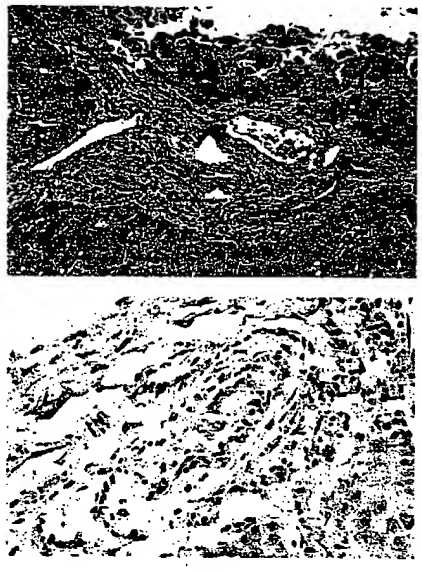

Fig 5—Histopathological studies.

Upper figure: 12 days—portal tract with most severe rejection (×100). Lower figure: 65 days—disruption of epithelium of septal bile duct with no evidence of rejection (×400).

Kupffer cell localisation of C3, and scant C4. Focal mild portal vein and hepatic arterial endothelial IgM deposits also were present. The diagnosis of mild humoral damage was made.

A wedge biopsy taken on day 12 showed Kupffer cell hypertrophy, mild centrilobular hepatocyte swelling, and cholestasis with a mild mononuclear portal and perivenular infiltrate (fig 5 upper). The infiltrate was comprised predominantly of T and NK cells, which were detected inside the basement membrane of a few small septal bile ducts and in the sinusoids. Subset analysis showed that about a third of portal T cells were CD4+, with the remainder presumably CD8+. Inflammatory or necrotising arteritis was not seen. Direct IF findings were qualitatively similar to those of the post-perfusion biopsy, but were less intense. No HBV antigens were detected and a PCNA labeling index in hepatocytes approached 35–40%. Diagnoses of regeneration and of mild cellular rejection (with a small humoral component) were made.

The single needle core of tissues obtained on day 24 was unremarkable except for mild cholestasis and Kupffer cell hypertrophy. Direct IF revealed striking decreases in all immunoglobulins and no detectable complement components. The diagnosis was mild cholestasis. The fourth liver biopsy on day 55 after an increase in total serum bilirubin to 78·7 mmol/L was also unremarkable except for Kupffer cell hypertrophy and a slight increase in sinusoidal mononuclear cells, with little or no portal or perivenular infiltrate and no evidence of inflammatory or necrotising vasculitis. Direct IF examination revealed a slight increase in sinusoidal immunoglobulin deposits with scant but detectable C1q. A diagnosis was made of sinusoidal reactivity with little evidence of rejection; minimum cholestasis was noted.

The fifth (wedge) biopsy was obtained intraoperatively on day 65, 3 days after the transhepatic cholangiogram which had led to a septic crisis. Cholestasis was conspicuous with

several bile infarcts, and focal mural necrosis of occasional septal bile ducts (fig 5 lower). Fewer than half the portal triads contained a mild mononuclear infiltrate, qualitatively similar in phenotypic composition to that seen earlier. More than 30% of bile ducts contained more T cells than NK cells inside the basement membrane, and these were associated with duct epithelial damage. There was no evidence of inflammatory, necrotising, or obliterative arteritis or coagulative vascular necrosis. Direct IF showed a sinusoidal pattern similar to that seen in the post-reperfusion sample, but with no arterial or vein deposits, consistent with the lack of vasculitis on light microscopy. Stains for HBV antigens were negative. A diagnosis of mild rejection, cholestasis, and bile infarcts, consistent with large duct obstruction, was made.

Endoscopic biopsy specimens of either the oesophagus, stomach, and/or duodenum were obtained on days 29, 30, 39, 43, and 55 because of complaints of anorexia and abdominal pain. Candida and severe cytomegalovirus oesophagitis were diagnosed on days 29 and 39, respectively.

Necropsy

The cause of death was diffuse subarachnoid haemorrhage and left uncul brainstem herniation, secondary to invasive aspergillosis in multiple sites in the left brain; haemorrhagic aspergillus lesions were also found in the left and right lungs, and left kidney. Other findings in the kidney were mild arterial nephrosclerosis, glomerular lobular accentuation, mesangial expansion and hypercellularity, and acute tubular necrosis. There was residual CMV oesophagitis and gastritis.

The hepatic arterial, portal venous, and vena caval anastomoses were patent. The biliary anastomosis was intact and patent, but the bileduct mucosa was dusky. Biliary sludge occupied the entire intrahepatic biliary tree, from the first-order branches through the extrahepatic ducts. Chemical studies of the material were characteristic of pigment sludge that, on histopathological examination, consisted of a large component (80%) of cellular debris. This finding was similar to that seen in past allografts with biliary stasis or obstruction. Multiple bile infarcts were present throughout the liver. There was no gross or histopathological evidence of inflammatory or obliterative arteriopathy of the deep hilar, peripheral, and peri-anastomotic graft arteries. There was no significant portal infiltrate in any of the multiple samples.

Discussion

We have reported previously that the incidence and management of liver allograft rejection were not substantially different in HIV-positive liver recipients than in those testing HIV negative.[21] Because our patient was immunocompetent before transplantation, the immunosuppression postoperatively was thought to be iatrogenic, not due to HIV. We believe that the four drug immunosuppressive regimen of FK 506, prednisone, PGE, and cyclophosphamide was effective in preventing both cellular rejection and the occlusive endotheliolitis attributed to antibody-mediated rejection in previously described baboon-to-human xenografts.[4,5]

The fatal outcome followed unrecognised biliary stasis with consequent diffuse damage of intrahepatic ducts, sludge formation, and bacterial infection. The raised alkaline phosphatase and jaundice were ascribed to rejection and treated as such despite the lack of histopathological evidence for this diagnosis. Similar technical and management difficulties of the biliary system, which ultimately proved correctable impeded early development of liver allotransplantation more than any other single factor.[22] It is likely that the biliary stasis found in our hepatic xenograft is preventable by stenting the biliary anastomosis with an exteriorised catheter that can also be used for cholangiography or irrigation postoperatively. However, we cannot exclude duct-specific immunologic injury or even chemical injury from a drug such as cyclophosphamide.

Our acknowledgement that failure in this case was probably technical permits an encouraging interpretation of other observations in this patient. The most important modification of immunosuppression must be restriction of cyclophosphamide to the perioperative period, as is necessary for prevention of antibody-mediated rejection of hamster organs transplanted to rats.[6,7] Because of the fear of organ rejection in our patient, this drug was continued for nearly two months with probable over-immunosuppression. However, the preformed lymphocytotoxic IgM antibodies present preoperatively in our patient's serum did not increase after transplantation, and no circulating cytotoxic IgG antibodies were ever measurable postoperatively. Diffuse IgM and IgG antibodies demonstrated in the xenograft biopsy sample by immunofluorescence at 12 days had largely disappeared by 24 days.

The liver xenograft is thought to have immunological advantages compared with other potentially transplantable organs, including greater resistance to humoral rejection and an unusual ability to take part in the induction of its own acceptance.[23] These advantages are explained by a high density of potentially migratory leukocytes of bone-marrow origin, including Kupffer cells, which are crucial to the development of systemic recipient chimerism.[24,25] Circulating baboon DNA was demonstrated by polymerase chain reaction after 35 days in our xenograft recipient, and at necropsy chimerism was confirmed in all tissues and organs tested. This finding after allo or xeno transplantation is construed as the first step toward chronic acceptance and ultimately donor-specific tolerance.[24]

An additional question largely answered by this single case is whether production by a liver xenograft of donor phenotype proteins and other synthetic products would result in a lethal incompatibility of metabolism in the recipient. The fall in serum uric acid and cholesterol concentration postoperatively to the low values that are normal for the baboon was an especially dramatic demonstration of the recreation by the xenograft of its own chemical environment and with no apparent adverse effects. As expected, the serum protein pattern of the recipient rapidly approached that of the baboon, including proteins involved in immune reactions or blood coagulation. The low serum albumin produced by the xenograft was well tolerated. These observations and those in rat recipients of hamster livers[26] suggest that donor-specific products of hepatic synthesis will not preclude liver xenotransplantation from a concordant species donor. It was also noteworthy that liver regeneration of the originally small xenograft took place at a similar time course described for the human liver.

Death at 70 days was too soon to allow a conclusion that the transplanted baboon liver could successfully resist infection with HBV. However, there was no evidence of the HBsAg or HBcAg in the transplant at necropsy. Infection of allografts with HBV has been recorded frequently during this time.[28] Since the baboon cannot be easily infected with HIV (if at all),[29] longer observation might have provided

data about HIV status of the chimeric baboon lymphodendritic cells of monocyte/macrophage lineage, which are thought to account for the post-transplant chimerism that is invariably found after successful hepatic allotransplantation. Evidence of chimerism was widespread in the xenograft recipient's organs. The crucial question will be whether HIV-resistant chimeric xenogeneic cells would have a survival advantage over infected autologous cells of the same lineage, and whether this could ultimately influence the course of HIV infection favourably. An additional question raised by the recent observation that FK 506 and cyclosporin interfere with HIV production and selectively inhibit growth of infected cells[30] is whether chronic immunosuppression with these drugs could have an antiviral effect. The long survival of several HIV-infected liver recipients is compatible with this possibility.[31]

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From The Lancet

Ocean travel

Life at the end of the nineteenth century is so eager and full of struggle, competition is so keen, the reward of successful effort so great, that most of us in every station neglect those precautions and habits upon which continued health chiefly depends. Prolonged and anxious intellectual labour, irregular hours, warm and ill-ventilated rooms, insufficient sleep and exercise, strain the nervous system, beget indigestion and innumerable physical evils. The physiological and prophylactic remedies of out-door exercise, rest, diet, rational amusement, which do so much to keep us in health, are not so generally utilised as their inestimable value deserves. Accordingly we approach the summer season jaded, worn, with nerves unstrung, appetite feeble, digestion faulty, sleep restless and insufficient. Rest is urgently demanded; the brain needs to lie fallow for a time that it may recuperate after its strenuous energy. Among the many means to which we may resort for restoration of physical and intellectual force I would unhesitatingly assign a high, perhaps the highest, place to ocean travel .... Life at sea is sufficiently full of incident to avoid the reproach of dullness. One meets with old friends or forms new acquaintances and a pleasant, leisurely intercourse permits the days to glide by without weariness. The sea air, always in circulation and charged with ozone, invigorates the exhausted system. Movement is not restrained. We feel the ship beneath us forging its way through the water, the deck affords us an ample promenade, the heaving surface of the ocean a picture full of charm. The pure salt breeze fans our cheeks, the porpoise or the shark claims a share of our attention, and as we traverse the ocean highway we meet other steamers sending up their clouds of smoke or sailing vessels with canvas spread to catch the wind.

(July 23, 1892)